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DEVELOPMENT OF SIMPLE SEQUENCE REPEAT MARKERS, FERTILITY STUDY AND SEED YIELD HERITABILITY ESTIMATES IN BERMUDAGRASS

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Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 2013

DEVELOPMENT OF SIMPLE SEQUENCE REPEAT MARKERS, FERTILITY STUDY AND SEED YIELD HERITABILITY ESTIMATES IN BERMUDAGRASS

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ACKNOWLEDGEMENTS

The author wishes to thank her major professor, Dr. Yanqi Wu, for his guidance and support during the course of the study and preparation of this dissertation. Thanks are also extended to Drs. Charles Taliaferro, Greg Bell, Michael Smith and Dennis Martin for serving as committee members and encouragement during the qualification examination and manuscript writing.

The author also thanks Dr. Timmy Samuels, Gary Williams, Sharon Williams and Pu Feng for laboratory technical support and assistance on field data collection.

The author wishes to express sincere gratitude to her parents for their understanding and support during her study aboard as a graduate student.

The author wishes to express appreciation to her husband, Michael Tamborski, for his encouragement and patience during her past two years of graduate study.

Acknowledgements reflect the views of the author and are not endorsed by committee members or Oklahoma State University.

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Date of Degree: DECEMBER, 2013

Title of Study: DEVELOPMENT OF SIMPLE SEQUENCE REPEAT MARKERS, FERTILITY STUDY AND SEED YIELD HERITABILITY ESTIMATES IN BERMUDAGRASS

Major Field: CROP SCIENCE

Abstract: The objectives of this study were to: develop and characterize genomic simple sequence repeat (SSR) markers in *C. transvaalensis* from its four SSR libraries enriched with [CA]n, [GA]n, [AAG]n, and [AAT]n; study selfing and outcrossing fertility in *C. dactylon* var. *dactylon* under open-pollinating conditions using SSR markers; and estimate heritability for seed yield and its two components, inflorescence prolificacy and seed set percentage in *C. dactylon* var. *dactylon*. There were 981 unique SSR markers developed and effective with reliable amplifications of targeted bands in *C. transvaalensis* donor DNA. Up to 93% of the 981 markers were polymorphic in eight *C. transvaalensis* plants and 544 markers were effective in all genotypes. A set of 917 markers amplified heritable alleles examined with six F_1 progeny of African bermudagrass parents 'T577' x 'Uganda'. In the fertility study, 25 clonal common bermudagrass plants were planted in a randomized complete block design with 3 replications at Perkins and Stillwater, OK. DNA samples were isolated from 1439 progeny germinated from open-pollinated seed of 25 seed parents. After comparing the progeny alleles to those of their respective maternal parents using 11 selected SSR markers, only two progeny were identified to be selfed, indicating an extremely high outcrossing rate of 99.86% in common bermudagrass under open-pollinating field conditions. The field trials were also used to collect data of seed yield, inflorescence prolificacy and seed set percentage in both 2012 and 2013. Half-sib families differed for all three traits, indicating the presence of additive gene action. Family × location effects were observed for seed set and seed yield. All three traits showed family × year interaction effects, while a significant family × location × year interaction existed in the two components. Narrow-sense heritability estimates (0.18 and 0.26-0.68) for seed yield, respectively based on variance component analysis and parent-offspring regressions, indicated a complex genetics of seed yield. Heritability estimates were moderate to relatively high for inflorescence prolificacy (0.30-0.55) and seed set (0.41-0.78). The results indicated that genetic improvement could be achieved if phenotypic selection is applied for seed yield components and conventional genotypic evaluation is needed for seed yield increase.

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CHAPTER I

GENERAL INTRODUCTION

RESEARCH OUTLINE

Bermudagrass, *Cynodon* (L.) Rich, is one group of long-lived perennial, warm-season, sod-forming grasses and has been widely used for turf and forage from tropical to warmer temperate regions of the world.

Cynodon genus belongs to tribe Cynodonteae Dumort., subfamily Chloridoideae Rouy, and family Gramineae Juss (Clayton et al., 1986). According to a widely adopted taxonomic revision by Harlan and colleagues (Harlan et al., 1970a), there are nine species and 10 varieties within *Cynodon* L. C. Rich. The base chromosome number per genome of the *Cynodon* species is nine (Forbes and Burton, 1963; Harlan et al., 1970b).

Cynodon dactylon (L.) Pers. is the most important species in the genus because of its prevalence, world-wide distribution, economic and ecological uses for livestock forage, as turf, and for soil stabilization and remediation. The species had been subdivided into six taxonomic varieties on the basis of geographic distribution and morphological, cytogenetic, and ecological characteristics (Harlan et al., 1970b). They are var. *afghanicus*, var. *aridus*, var. *coursii*, var. *dactylon*, var. *elegans* and var. *polevansii*, having diploid ($2n=2x=18$), tetraploid ($2n=4x=36$) an

hexaploid (2*n*=6*x*=54) cytological forms (Harlan et al., 1970; Harlan et al., 1970b; Hanna et al., 1990).

Cynodon dactylon var. *dactylon*, also called common bermudagrass, is prominent due to its cosmopolitan, ubiquitous and remarkable distribution characters. Plants of var. *dactylon* vary from small, fine leaf texture suitable for putting greens to large, coarse, robust types used for hay or grazed as pasture grass (Harlan and de Wet, 1969). There were three major races of var. *dactylon* described by Harlan and de Wet (1969) as tropical, temperate and seleucidus races based on adaptation characteristics and the centers of diversity. Sometimes the races are sympatric. Variety *dactylon* is tetraploid (2*n*=4*x*=36), which has been used in interspecific hybridization with *Cynodon transvaalensis* to produce hybrid turfgrass cultivars.

C. transvaalensis, commonly called 'African bermudagrass', is a turf-forming species in the genus and easily distinguished from the other species based on its unique morphological traits. The species is narrowly endemic to Transvaal, Orange Free State and the north-central Cape Province of South Africa (de Wet and Harlan 1971; Harlan et al. 1970a). Diploid (2n=2x=18) African bermudagrass generally crosses readily with tetraploid (2n=4x=36) and hexaploid (2n=6x=54) common bermudagrass (Harlan et al., 1970a; Taliaferro et al. 2006). The greatest value of the taxon emanates from its use in hybridizations with *C. dactylon* var. *dactylon* to produce vegetatively-propagated F_1 hybrid turf cultivars (Hein, 1953; Burton, 1991; Taliaferro et al., 2004). Genetic variability in African bermudagrass continues to be used in breeding new superior interspecific hybrid turf cultivars (Kenworthy et al. 2006). Although African bermudagrass remains important to the turf industry, avaialble molecular markers to assist conventional breeding are limited in the species up to date.

Simple sequence repeat (SSR) markers are one type of polymerase chain reaction (PCR) based molecular marker systems. There have been other PCR- based marker systems, including DNA

amplification fingerprinting (DAF), random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP) used in the past (Caetano-Anolles et al. 1995; Assefa et al. 1999; Zhang et al. 1999; Roodt et al. 2002) in bermudagrass such as genetic diversity analysis and characterization of bermudagrass germplasm accessions. Due to the co-dominant nature of SSR markers, this molecular marker system has become a powerful marker tool that can easily identify homozygous and heterozygous locus compared to other types of markers. It has been reported about available SSR markers being used in the identification of bermudagrass cultivars (Wang et al. 2010). Development of SSR markers in the species of interest could be contributing to genetic map construction, investigation of associations between traits and markers and marker-assisted selection for breeding programs.

Sexual reproduction as the predominant mode of production in *C. dactylon* is outcrossing due to cross-pollination and self-incompatibility characteristics, which have been well documented. Burton and Hart (1976) indicated that high levels of self-incompatibility exist in *Cynodon dactylon* (L.) Pers. germplasm. Richardson et al. (1978) reported that there was low self-pollination seed set average ranged from 0.10 to 8.09% from 30 progeny clones with the average less than 1% for the most clones. To determine the percent of self-pollinated seed set, inflorescences were individually enclosed within small weather-proof pollinating bags. There is no available information to quantify self-pollination behavior under naturally open-pollinating field conditions. SSR marker system could be used as an accurate and efficient tool to provide such information assisting bermudagrass breeding in field conditions for germplasm improvement, hybrid development and formation of synthetic cultivars.

As another interest in bermudagrass breeding, seed yield has become one major trait targeted by breeders and industry to develop desirable seeded bermudagrass cultivars competing with clonal cultivars (Taliaferro, 2003) over the past quarter century. According to Ahring et al. (1974), seed-propagated bermudagrass cultivars were needed because commercial planting equipment and

establishment technology for vegetative propagation were not effective for confined areas, including home lawns and the areas on steep embankments such as dam faces or roadsides. Additionally, seeding requires less time and physical labor than vegetative propagation. Heritability is the amount of phenotypic variation due to genotypic variation. Knowledge of heritability will increase breeding efficiency by aiding in planning for each cycle of selection because if heritability is low and environmental variation high, then more testing in multiple environments is required. There has been some limited information reported on seed yield heritability estimates by Cluff and Baltensperger (1991). They indicated that seed yield was 42% partially determined by additive gene action, suggesting that phenotypic selection methods could be effective in increasing seed yield. The realized heritability estimates for the components related to seed yield ranged from 0.33 to 0.94. However, due to low seed yield and seed set in the study, the estimates could be substantially biased. Providing more robust information on seed yield and its components heritability estimates is needed, which would effectively direct the breeding strategy and accelerate the progress for the improvement of the trait.

Thus, the three components designed for this research are development of SSR markers in African bermudagrass, fertility study in common bermudagrass under naturally open-pollinating conditions and heritability estimates for seed yield and its components in bermudagrass.

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CHAPTER II

DEVELOPMENT AND CHARACTERIZATION OF GENOMIC SSR MARKERS IN *CYNODON TRANSVAALENSIS* BURTT-DAVY

ABSTRCT

African bermudagrass, *C. transvaalensis*, is an economically important warm-season turf grass species mainly used for hybridizations with common bermudagrass *C. dactylon* var. *dactylon* (L.) Pers. in the development of superior interspecific hybrid turf cultivars. However, few simple sequence repeat (SSR) markers are currently available in the taxon. Accordingly, the major objective of this study was to develop and characterize a large set of SSR markers. Genomic DNA of C. transvaalensis '4200TN 24-2' from an Oklahoma State University (OSU) turf nursery was extracted for construction of four SSR genomic libraries enriched with [CA]n, [GA]n, [AAG]n, and [AAT]n as core repeat motifs. A total of 3064 clones were sequenced at the OSU core facility. The sequences were categorized into singletons and contiguous sequences to exclude the redundancy by CAP3 program. From the two sequence categories, 1795 SSR loci were identified. After excluding duplicated SSRs through a comparison with previously developed SSR markers using a nucleotide basic local alignment tool, 1426 unique primer pairs (PPs) were designed by SSR Locator software. Out of the designed PPs, 981 (68.8%) were effective with reliable amplifications of targeted bands in the donor DNA. Polymorphisms of the SSR PPs tested in eight C. transvaalensis plants were at 93% and 544 markers were effective

all genotypes. Inheritance of the SSRs was examined in six F_1 progeny of African parents 'T577' x 'Uganda', indicating 917 markers amplified heritable alleles. The SSR markers developed in the study are the first large set of co-dominant markers in African bermudagrass, and should be highly valuable for genetic and breeding research of the species.

INTRODUCTION

C. transvaalensis is a turf-forming perennial species, which is endemic to damp habitats in the southern and western Transvaal, Orange Free State and the north-central Cape Province of South Africa (de Wet and Harlan 1971; Harlan et al. 1970). Commonly called 'African bermudagrass', the taxon is distinct from other *Cynodon* species because of its distinct and unique morphological characteristics such as small plant size, erect and fine linear leaf blades, yellowish green color, and limited geographic distribution (de Wet and Harlan 1970). Diploid ($2n=2x=18$) African bermudagrass generally crosses readily with tetraploid ($2n=4x=36$) and hexaploid ($2n=6x=54$) common bermudagrass (Harlan et al., 1970a; Taliaferro et al. 2006). Though African bermudagrass clonal varieties have had limited commercial use for turf, the greatest value of the taxon emanates from its use in hybridizations with *C. dactylon* var. *dactylon* to produce vegetatively-propagated F1 hybrid turf cultivars (Hein, 1953; Burton, 1991; Taliaferro et al., 2004). Most of the contemporary industry standard clonal turf bermudagrass cultivars are interspecific hybrids from crosses of *C. transvaalensis* and *C. dactylon* parent plants. 'Tifway' and 'Tifgreen' bred at the USDA ARS Coastal Plains Experiment Station at Tifton, Georgia and 'Midlawn' from the Kansas State University are triploid hybrids ($2n=3x=27$) whereas 'Patriot' from the Oklahoma State University is a tetraploid interspecific hybrid ($2n=4x=36$) (Alderson and Sharp 1994; Taliaferro et al. 2006). Patriot bermudagrass is a clonal F1 hybrid of a *C. transvaalensis* ($2n=2x=18$) selection and 'Tifton 10', a hexaploid ($2n=6x=54$) *C. dactylon* plant from China (Taliaferro et al. 2006; Burton 1991). Genetic variability in African bermudagrass continues to be used in breeding new superior interspecific hybrid turf cultivars (Kenworthy et al.

2006). Although African bermudagrass remains important to the turf industry, the development and use of molecular markers are limited in the species up to date.

Polymerase chain reaction (PCR) based molecular marker systems have been used to characterize genetic diversity or relatedness of bermudagrass germplasm accessions including African bermudagrass (Caetano-Anolles et al. 1995; Assefa et al. 1999; Zhang et al. 1999; Roodt et al. 2002). The most common marker systems used have been DNA amplification fingerprinting (DAF), random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP). The genetic relatedness of fourteen *C. transvaalensis* accessions, two hexaploid *C. dactylon* var. *dactylon* accessions and two interspecific tetraploid F1 hybrids was examined using AFLP markers by Wu et al. (2005). Their results indicated the presence of substantial genetic diversity in *C. transvaalensis* that could be exploited both in intra- and interspecific breeding improvement. These techniques are all PCR-based systems and can rapidly generate a large group of polymorphic bands with a small amount of DNA as reaction templates. However, they are dominant marker systems which have limitations in the identification of heterozygous genotypes from homozygous genotypes. Simple sequence repeat (SSR) markers are a PCR-based molecular marker system. Because of their co-dominant nature, SSR markers are more powerful compared to other PCR-based marker systems, especially in the identification of bermudagrass cultivars (Wang et al. 2010), genetic map construction, and marker assisted breeding.

Genomic SSR markers have the advantages of being highly polymorphic and evenly interspersed through the genome of both prokaryotic and eukaryotic organisms when compared with expressed sequence tag (EST) SSR markers (Tóth et al. 2000). The SSR marker system has been developed and widely used in many plant species, including major crops such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), maize (*Zea mays*) and sorghum (*Sorghum bicolor*) (Senior et al. 1996; Roder et al. 1998; Temnykh et al. 2000; Bhattramakki et al. 2000).

A small number of SSR markers are available in African bermudagrass, which were transferred from sorghum and developed from expressed sequence tags (Harris-Shultz et al. 2010; Tan et al. 2012). The available SSR markers in bermudagrass have been utilized in clonal turf bermudagrass cultivars identification (Wang et al. 2010), linkage map construction (Harris-Shultz et al. 2010) and SSR transferability study (Harris-Shultz et al. 2012). Compared to EST SSR markers, genomic SSRs are more polymorphic, making them highly valuable for construction of genetic maps and investigation of associations between traits and markers. However, SSR markers developed from genome sequences of African bermudagrass are not available. Accordingly, the objectives of this study were to develop and characterize a large set of simple sequence repeat markers in *C. transvaalensis* from its SSR-enriched genomic libraries.

MATERIALS AND METHODS

Plant materials and genomic DNA isolation

C. transvaalensis '4200TN 24-2', an OSU selection, was used to isolate DNA for constructing SSR-enriched genomic libraries. The '4200TN 24-2' DNA was also used for the initial screening of designed SSR primers. A diversity panel (Panel 1) encompassing eight *C. transvaalensis* plants from different origins was used to test marker polymorphism and analyze genetic diversity (Table 2.1). These plants have substantial dissimilarity based on a previous study by Wu et al. (2005) and by their geographic origins. Panel 2 consisting of maternal parent 'T577', paternal parent 'Uganda' and six F1 progeny from crosses of the parents was used to examine the reliability of SSR primer pairs by observing the transmission of SSR alleles from parents to progeny. All plants were grown in a greenhouse at the OSU Agronomy Research Station and appeared healthy at the time of sampling. Approximately 1.5g of leaf tissue from each separately potted plant was collected to extract genomic DNA using the CTAB method (Doyle et al. 1990). Genomic DNA was quantified using a NanoDrop ND-1000 Spectrophotometer

(Nanodrop products, DE, USA) and quality was checked by 1% agarose gels. Based on the measured quantity, DNA samples were diluted to 10ng/ µl as working templates for PCR amplification.

Construction of SSR-enriched genomic libraries and DNA sequencing

Approximately 100 µg of genomic DNA from '4200TN 24-2' was sent to Genetic Identification Services (GIS, Chatsworth, CA, USA) for constructing four SSR-enriched genomic libraries with respective core repeat sequences [CA]n, [GA]n, [AAG]n, and [AAT]n. According to manufacturer's protocol, genomic DNA was partially restricted with a cocktail of seven blunt-end cutting enzymes (*Rsa*I, *Hae*III, *Bsr*B1, *Pvu*II, *Stu*I, *Sca*I, *Eco*RV) (GIS, Chatsworth, CA, USA). DNA fragments in the size range of 300 to 750 bp were adapted and subjected to magnetic bead capture (CPG, Inc., Lincoln Park, New Jersey) using biotinylated capture molecules. The libraries were prepared in parallel using Biotin-(AAAC)8, Biotin-(CATC)8, Biotin-(TACA)8, and Biotin-(TAGA)8 as capture molecules in a protocol provided by the manufacturer. Captured molecules were amplified and restricted with *Hind*III to remove the adapters. The resulting fragments were ligated into the *Hind*III site of pUC19 plasmid vector in *Escherichia coli*.

The transformants were recovered by mixing 10 µl of each library with 30 µl of Genomic Grate Culture Media SOC Sterilized Broth (Growcells, Irvine, CA) and spread out onto LB/AMP (100 µg/ml) / X-GAL (80 µg/ml) / IPTG (50 µM) plates (Teknova Inc., CA, USA), using a sterilized bacterial spreader to make an even distribution of the mixture for blue-white screening. Plates were incubated overnight at 37 ℃ without light and immediately placed in 4 ℃ for at least two additional hours to enhance blue color. White colonies (DNA fragment containing clones) were picked with sterilized toothpicks and placed into 96 well blocks, containing 1 ml LB broth with Ampicillin (100 µg/ml) in each well. For each library 766 clones were picked and placed in

eight 96-well culture blocks with one positive (a blue colony) and one negative control (one plain sterilized toothpick). Blocks were covered with breathable film and grown overnight (18h) at 37 ℃ with shaking at 250 rpm. The other eight blocks containing 50 μl of 30% glycerol in each well were mixed with 50 μl of overnight culture, sealed with aluminum tape and stored at -70 ℃ as stocks for each library. The remaining solution in the overnight grown blocks was spun down in a centrifuge at a speed of 2500 $\times$g for 7 minutes. The solution in each well was decanted, and the culture blocks were covered with aluminum tape then sent to the OSU Recombinant DNA / Protein Resource Facility (OSU Core Facility) for plasmid preparation and sequencing.

DNA sequencing of picked recombinant clones was performed using an ABI BigDye® Terminator v1.1 Sequencing Kit and analyzed on an ABI Model 3730 DNA Analyzer (Applied Biosystems Inc., CA, USA) at the OSU Core Facility. Quality of all DNA sequences was analyzed by Sequence Scanner v1.0 (Applied Biosystems Inc., CA, USA). After trimming off the vector sequences, all the sequences were placed in a fasta file for further analysis.

Detection of SSR sequences and design of non-redundant SSR primers

The fasta file containing all trimmed sequences was analyzed with the CAP3 program (Huang et al. 1999) to categorize the sequences into contiguous sequences (contigs) and singletons. The program parameters were set with the default values while ‘overlap identity cutoff = 95’ and ‘clipping range = 6’ were selected for the best multiple sequence alignments. The two categories of sequences were run separately with the SSR Locator program (da Maia et al. 2008) for SSR detection and primer designing. The parameters for SSR detection were set as: 5-di-, tri-, tetra-; 4-penta-, hexa-; 3-hepta-, octa-, nona-, and decanucleotide repeats. The parameters for the primer design were the following: amplicon size in 140-350 bp; primer length 18 to 22 bases with 20 as the optimum; the annealing temperature 55 ℃ to 61 ℃ with the optimum of 59 ℃; GC clamp 0; G/C content 45% to 50%; start and end point automatic scan and end stability at 250.

Forward primers designed by SSR Locator were attached with M13 (5'-CACGACGTTGTAAAACGAC-3') at the 5' end of each forward primer sequence (Integrated DNA technologies, Inc., IA, USA) for DNA amplification detection purpose.

To prevent any redundancy of designed primers with previously developed bermudagrass EST-SSR primers (Tan et al. 2010) and *C. dactylon* genomic SSR primers (manuscript in preparation), a comparison was performed between the forward and reverse primers described above and all primer sequences developed from the four '4200TN 24-2' genomic libraries in this study. Each set of sequences was compared by aligning two or more sequences together using a specialized nucleotide basic local alignment search tool (bl2seq) (BLAST, http://blast.ncbi.nlm.nih.gov/). The program was optimized for highly similar sequences (megablast) with default parameters with the word size algorithmic parameter changed from 28 to 16 based on the size of primers (18-22 bp).

PCR amplification and gel electrophoresis for screening the designed SSR PPs

The non-redundant SSR primers were tested for their effectiveness by target PCR amplifications of the *C. transvaalensis* '4200TN 24-2' genomic DNA used for the library construction. PCR reactions were performed on Applied Biosystems 2720 thermal cyclers (Applied Biosystems Inc., CA, USA). Each reaction contained 4.3 µl nuclease free water, 1.0 µl 10×reaction buffer, 0.6 µl 25 mM MgCl2, 0.2 µl 10 mM deoxynucleoside triphosphate (dNTP), 1.34 µl 1 pmol/µl forward primer, 1.34 µl 1 pmol/µl reverse primer, 0.2 µl 1 µM IR-M13 forward primer, 0.03 µl 0.5X Taq DNA polymerase and 1.5 µl of 10 ng/µl template DNA. Thermal cycler parameters set as 5 min at 94 ℃, 14 cycles of 20 s at 94 ℃, 1 min at 58 ℃ and 30 s at 72 ℃, then 28 cycles of 20 s at 94 ℃, 1 min at 55 ℃ and 30 s at 72 ℃, and followed by 10 min extension at 72 ℃ followed Wu and Huang (2008). The amplified PCR products were placed on ice until gel loading or stored at 4 ℃.

The initial test of the unique designed SSR PPs from all four libraries were performed on AdvanCE TM FS96 (Advanced Analytical Technologies, Inc., IA, USA) for the electrophoresis of PCR amplified products. This equipment used a 96-capillary array and an intercalating dye for amplicon detection using an LED light source. Due to the utilization of the intercalating dye, there was no denaturation needed prior to PCR products analysis. Nuclease free water was added to a final volume of 30 µl per well of a 96-well PCR plate, which was placed in the equipment before running. Each capillary was loaded with two size markers (35bp and 500bp) and a standard curve was performed on each batch of a new gel. The marker ladders were used to align each capillary for analysis using Pro-size DNA analysis software (Advance Analytical Technologies, Inc., IA, USA). Parameters for each of the simultaneous 96 wells on the AdvanCE FS96 were in the order described as follows: stage out, gel prime, pre-run at 9 kilovolts (KV) for 30 seconds, base pair marker plate injection at 5 KV for 25 seconds, no water dip, vacuum sample injection for 45 seconds, removal of sample and marker trays, then capillary electrophoresis separation at 9 KV for 95 min. The SSR bands with expected amplified product size and reproducible in two replications were considered effective markers.

Inheritance evaluation and diversity analysis

Two experiments were performed to characterize the effective non-redundant SSR PPs evaluated from the initial screening test. Experiment 1 was to characterize allele polymorphism and usefulness of the effective PPs for genetic diversity evaluation in Panel 1. For diversity analysis, all reliable bands were scored as presence (1) or absence (0) among results from eight transvaalensis genotypes showing by each primer pair input into a binary matrix. Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) version 2.10 software (Rohlf, 2002) with SIMQUAL module was used to analyze the data set to generate a genetic similarity (GS) coefficients matrix. With the GS matrix, a dendrogram was constructed using unweighted pair group method arithmetic average (UPGMA) to determine genetic relationships among the eight

genotypes. Experiment 2 was used to examine the reliability and transmission of alleles amplified by each primer pair from parents to their progeny in Panel 2. The clean and reliable PCR bands in the two experiments were visually scored using SagaGT 3.3 (LI-COR Inc., Lincoln, NE, USA).

RESULTS

Sequencing of selected clones, design and initial screening of SSR primers

For each of the four SSR enriched libraries, 766 selected clones that showed white color were sequenced resulting collectively in 3050 trimmed sequences available for SSR marker development. After all the sequences were assembled using the CAP3 program, there were 326 contigs and 1931 singletons. Of the 2257 sequences of contigs and singletons running through the SSR Locator, 1795 (79.5%) including 282 contigs and 1513 singletons were identified to have SSRs. Of the two groups of SSR-containing sequences, 249 and 1184 non-redundant PPs were designed respectively. Comparing the 1433 unique PPs with our unpublished C. dactylon SSR primer sequences and published EST-SSR primer sequences (Tan et al. 2012), seven matched PPs (i.e., duplicated SSRs) of this experiment were excluded. The remaining 1426 SSR PPs were used for PCR amplification of '4200TN 24-2' DNA as initial screening, resulting in 981 (68.8%) PPs that were effective and reliable in the amplification of target bands. Among the four libraries, efficiency differed in the development of reliable SSR markers. The AAG-enriched library was the most efficient (72.8%) and contributing 177 effective markers. Efficiencies of the CA, GA, and AAT-enriched libraries were respectively 71.9%, 69.7%, and 57.5% (Table 2.2).

Characterization of effective SSR markers

Among the 981 unique and effective SSR markers, 754 (76.9%) were of perfect repeat types, ranging from 87.0% to 62.4% in the four libraries. The AAG-enriched library had the highest percentage of perfect SSRs among the four libraries. The other 227 (23.1%) SSRs were of

compound types which had more than one core repeat. The percentage of compound SSRs in each library varied from 13.0 to 37.6% with the highest in the CA-enriched library.

The number and frequency for perfect, compound and other SSR motifs in each of the four libraries are presented in Figure 2.1(a-d). The CA-enriched library consisted of 58.8% CA/GT and AC/TG repeat motifs, whereas the GA-enriched library had 78.8% TC/AG and CT/GA repeat motif sequences. In the AAG-enriched library, repeat motifs AAG/TTC, CTT/GAA and AGA/TCT accounted for 29, 27 and 22%, respectively. Over the four libraries, the predominant motifs of SSR markers were dinucleotide repeats (CT/GA, TC/AG, AC/TG and CA/GT), representing 50%. The expected trinucleotide repeats (AAG/TTC, AAT/TTA, TAA/ATT, CTT/GAA, TAT/ATA, AGG/TCC and AGA/TCT) accounted for 24% of all SSR motifs.

Evaluation of inheritance of SSR marker alleles

Testing the transmission of SSR alleles from parent plants to their progeny provided valuable information on the reliability and heritability of effective SSR markers. All 981 effective SSR markers were tested through panel 2 consisting of maternal parent 'T577', paternal parent 'Uganda' and six F1 progeny derived from crossing the two (Figure 2.2). Among all effective markers, 600 SSRs (61.2%) exhibited strictly heritable bands in the parents-progeny test panel. These markers were effective in both parents and showed heritable amplifications from both parents into their progeny. Out of the 600 strictly heritable SSRs, 125 markers amplified monomorphic bands while the other 475 markers were polymorphic among the parents and segregating in the progeny (e.g. primer combination 1983/1984 in Figure 2.2). The number of markers and percentages in each of the four libraries are given in Table 3. There were 64 (6.5%) SSR primers that showed non-target amplifications in the panel. Among the remaining 317 heritable SSR markers, 67 markers amplified effective alleles only in one parent and some

progeny (e.g., primer combinations 1991/1992 in Figure 2.2). The other 250 SSR markers showed a missing parental allele in at least one progeny (e.g. primer combinations 1987/1988 and 1999/2000 in Figure 2.2). Thus, the inheritance evaluation examined that 917 SSR markers amplified true heritable alleles in the testing panel. All the sizes of the heritable alleles in 'T577' and 'Uganda' were recorded (ESM). The 600 strictly heritable markers were noted 'HP' as heritable polymorphic and 'HM' as heritable monomorphic. The rest of the SSR markers were scored with heritable allele sizes in 'T577' and 'Uganda', otherwise 'N/A' was recorded when the markers were not effective.

Polymorphism evaluation of SSR markers

The polymorphisms of all effective SSR markers were evaluated by counting allele number and scoring allele size amplified in panel 1 of 8 *transvaalensis* plants (Figure 2.3, ESM). The number of alleles amplified per SSR primer pair for individual genotypes of the panel varied from 0 to 4. Among the 981 effective SSR PPs, 32 (3.3%) were monomorphic among the eight genotypes. Among the 544 (55.5%) SSR PPs effective in all 8 genotypes, 506 were polymorphic with a polymorphism rate of 93%. The number of distinct alleles amplified by each of the SSR PP among the eight genotypes ranged from 1 to 10 with an average of 4.

Analysis of genetic diversity of eight *C. transvaalensis* plants

A total of 3971 amplified alleles from 981 SSR markers were used in the analysis of genetic diversity among the eight African bermudagrass genotypes tested for SSRs polymorphism. The SSR markers clearly separated each genotype with their respective similarity coefficients (0.53-0.95) between each two of the eight genotypes. PI290812 (41-222) and PI290894 (Sekaapploss fine) were clustered together with the greatest genetic similarity coefficient 0.95 (Figure 2.4). The results demonstrated that the 8 *C. transvaalensis* plants were genetically distant except PI290812 and PI290894 both originally from South Africa.

DISCUSSION

Efficiency of genomic SSR marker development

Because whole genome sequences are still unavailable in African bermudagrass, using SSR-enriched genomic libraries provided an efficient means to develop SSR markers. CAP3 program analysis indicated that 2257 sequences (74%) were unique of all 3050 trimmed clones. Of these 2257 unique clone sequences, 79.5% of both the contiguous sequences and singletons contained SSR repeats. Compared with other studies reporting SSR-enriched libraries in plants, the proportion (79.5%) of SSR-containing clones in *C. transvaalensis* was similar to the results obtained in switchgrass (*Panicum virgatum*) (83.5%, Wang et al. 2011), eggplant (*Solanum melongena*) (81.7%, Nunome et al. 2009) and zoysiagrass (*Zoysia japonica*) (81.7%, Cai et al. 2005) and higher than Italian ryegrass (*Lolium multiflorum*) (67.6%, Hirata et al. 2006). The total number of effective primer pairs in *C. transvaalensis* '4200TN 24-2' DNA donor were 981, 32.2% of the 3050 trimmed clone sequences, which is close to the 33.8% effective markers of total clones found in switchgrass (Wang et al. 2011). These results indicated that the use of SSR-enriched libraries was an effective method in developing a large set of SSR markers in *C. transvaalensis*.

The most common SSR motifs in the genome of *Arabidopsis thaliana* are AT, GA, CA of dinucleotide repeats, and AAG, ATC, AAC and AAT as trinucleotide repeats. The most frequent dinucleotide repeats in the four SSR-enriched genomic libraries in *C. transvaalensis* are GA (32%) and CA (18%). This result is in agreement with previous studies in other grasses, such as switchgrass (Wang et al. 2011), zoysiagrass (Cai et al. 2005) and Italian ryegrass (Hirata et al. 2006). This study further indicates that GA- and CT-enriched genomic SSR libraries are the most efficient for the development of SSR markers in the grass species.

Inheritance evaluation of SSR markers

SSR markers can be used for multiple applications in bermudagrass genetic analysis and molecular breeding, such as germplasm diversity analysis, cultivar characterization and identification, genetic mapping, QTL identification and eventually marker assisted selection. Inheritance evaluation of SSR markers is one essential procedure for testing the reliability of SSR markers before they are applied to various genetic investigations. In a study of switchgrass SSR markers-based linkage map construction, Liu et al. (2012) found that there were non-heritable bands in the genomic SSR markers previously reported by Wang et al. (2011). In this study, primer pair 1991/1992 amplified a band in 'T577' on a diversity gel image (Figure 2.3). When compared with the progeny test gel image (Figure 2.2) this band was not inherited by the progeny. The result may further suggest that some PCR bands were non-heritable and without progeny testing would be identified as effective alleles. It is apparent that inheritance evaluation is valuable and important before markers are reported and applied to linkage map construction. In our study, 981 SSR markers were examined in 'T577' (maternal), 'Uganda' (paternal) and six F1 progeny. The evaluation resulted in 917 markers which amplified heritable alleles. Of these 917 SSR markers, 600 SSR markers had strict heritability from the two parents to their progeny, which included 475 (79.2%) polymorphic markers that were highly valuable for molecular mapping in the population of 'T577' and 'Uganda'. For the remaining heritable markers, 76 were only effective in one parent indicating that the markers were genotype dependent. The other 241 markers amplified alleles in parents but not in all progeny. The results may be derived from PCR failures if both alleles are not shown in progeny or likely mutations occurred in annealing sequences if only one parental allele was present and the other absent.

SSR markers polymorphism in *C. transvaalensis* genetic diversity evaluation

Eight *C. transvaalensis* genotypes were used for SSR markers polymorphism evaluation. Out of 544 consistently effective SSR markers across the eight genotypes, 506 (93%) were polymorphic. This finding indicated that genomic SSR loci were highly variable and informative

among the eight genotypes that were chosen to represent the wide diversity in *C. transvaalensis*. The remaining 437 PPs had at least one genotype with no effective amplification in the panel perhaps resulting from mutations in the annealing sequences of SSR primers among their diverse genotypes. Similar results were reported from a phylogenetic analysis by genomic SSR markers in wheat (Gadaleta et al. 2011), indicating that the level of polymorphism of the markers was genotype dependent. Our dendrogram (Figure 2.4) with genetic similarity coefficients ranging from 0.53 to 0.95 among the eight genotypes showed a wide diversity range within *C. transvaalensis*. This diversity is consistent with the results of Wu et al. (2005). In this study using SSR markers, a genetic similarity coefficient of 0.95 between PIs 290812 and 290894 was greater than the similarity coefficient of 0.79 with AFLP markers (Wu et al. 2005).

To summarize, the present study is the first dedicated investigation in the development of a large set of 981 unique SSR markers from four SSR-enriched genomic libraries in *C. transvaalensis*. Most of the SSR PPs amplified heritable alleles. The SSR markers were highly effective in genetic diversity analysis across a panel of *C. transvaalensis* plants with different geographic origins. Thus, the genomic SSR markers developed from this study should be highly valuable for multiple genetic research uses and molecular breeding in African bermudagrass.

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Table 2.3. Inheritance of 981 unique SSR markers for each of four SSR-enriched genomic libraries.

Table 2.1. Eight *C. transvaalensis* plants with their PI numbers, other IDs, origins and references.

No.	PI	Other ID	Origin	Reference
1	PI 291981	K5996	Ethiopia	NPGS‡
2	PI 290894	Sekaapploss fine	South Africa	Wu et al. 2005
3	PI 286584		India	NPGS
4	PI 615161		Israel	NPGS
5	PI 290874	Uganda	Egypt	Wu et al. 2005
6	PI 290812	41-222	South Africa	Wu et al. 2005
7		T577	Lesotho	Wu et al. 2005
8		4200TN 24-2	OSU Turfgrass Nursery	

‡ NPGS= National Plant Germplasm System

Table 2.2. Efficiency of SSR markers designed for each of four SSR-enriched genomic libraries.

Library	Clones sequenced	SSR sequences identified	Non-redundant primer pairs designed	Effective SSR markers	Efficiency (%)
(CA)n	766	492	388	279	71.9
(GA)n	766	678	555	387	69.7
(AAG)n	766	314	243	177	72.8
(AAT)n	766	311	240	138	57.5
Four libraries	3064	1795	1426	981	68.8

Table 2.3. Inheritance of 981 unique SSR markers for each of four SSR-enriched genomic libraries.

Library	SSR markers analyzed	Strictly heritable markers with monomorphic bands	Strictly heritable markers with polymorphic bands	No target reaction	Non-strictly heritable amplified SSR markers
(CA)n	279	26(9.3%)	136(48.7%)	15(5.4%)	102(36.6%)
(GA)n	387	38(9.8%)	192(49.6%)	28(7.3%)	129(33.3%)
(AAG)n	177	45(25.4%)	76(42.9%)	10(5.7%)	46(26.0%)
(AAT)n	138	16(11.6%)	71(51.4%)	11(8.0%)	40(29.0%)
Four libraries	981	125(12.7%)	475(48.4%)	64(6.5%)	317(32.3%)

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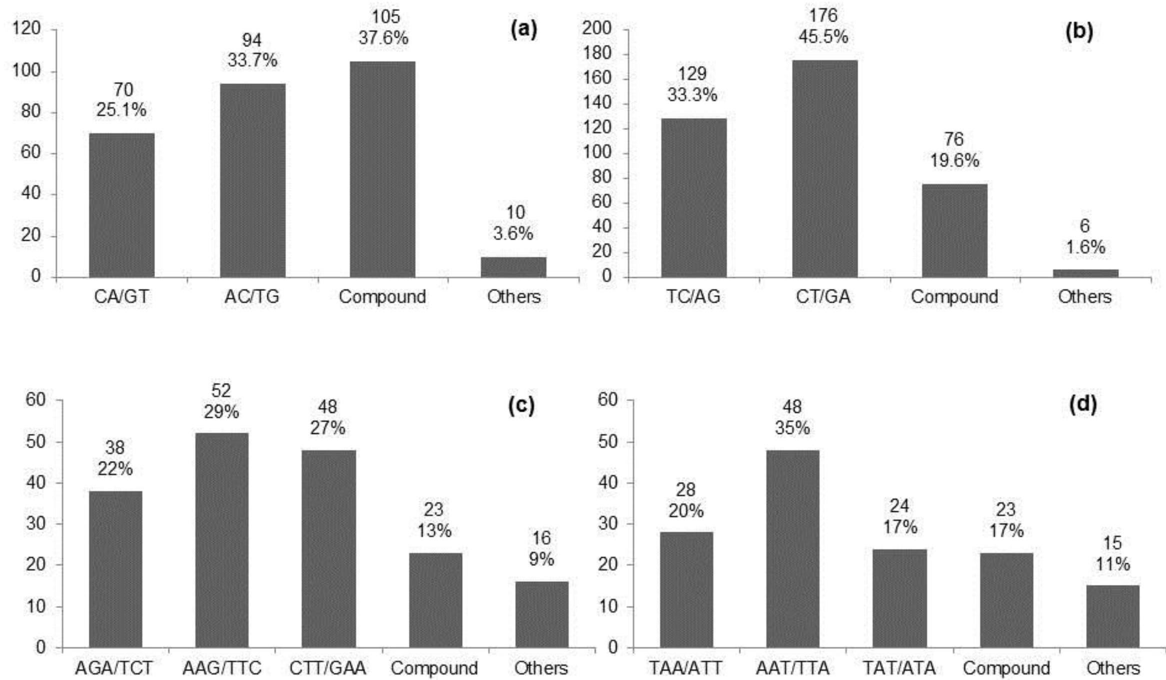
Figure 2.1. Number and frequency for perfect, compound and other repeat motif types in four SSR-enriched genomic libraries. (**a**) CA-enriched library, (**b**) GA-enriched library, (**c**) AAG-enriched library, and (**d**) AAT-enriched library.

Figure 2.2. One inheritance evaluation gel image by eight SSR primer pairs. Primer information was listed with their identification numbers at the bottom and standard size markers were given on both sides of the gel image. The order of DNA samples for each SSR primer pair from lane 1 to 8 was 'T577', 'Uganda' and their six hybrid progeny. The boxes indicated the each marker target amplification regions for scoring heritable alleles respectively.

Figure 2.3. One polymorphism evaluation gel image by eight SSR primer pairs. Primer identification numbers were listed at the bottom and standard size markers were given on both sides of the gel image. The order of DNA samples from lane 1 to 8 for each primer pair was PIs 291981, 290812, 290894, 286584, 'T577', 615161, 290874 and '4200TN 24-2'.

Figure 2.4. The tree plot constructed using unweighted pair group method with arithmetic average for eight *C. transvaalensis* genotypes based on 3971 amplified SSR bands.

Figure 2.1. Number and frequency for perfect, compound and other repeat motif types in four SSR-enriched genomic libraries. (**a**) CA-enriched library, (**b**) GA-enriched library, (**c**) AAG-enriched library, and (**d**) AAT-enriched library.



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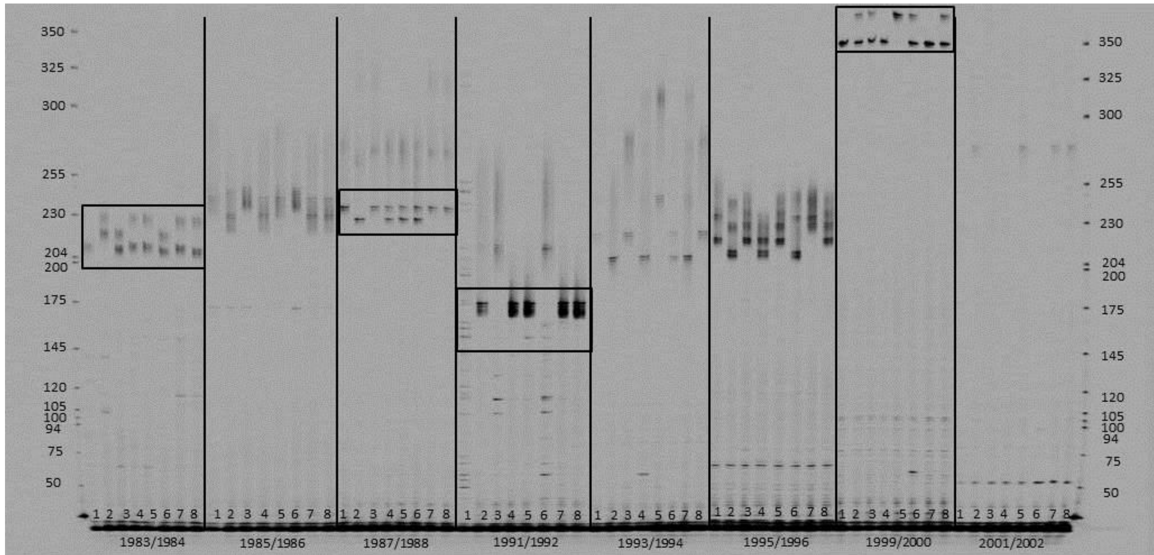

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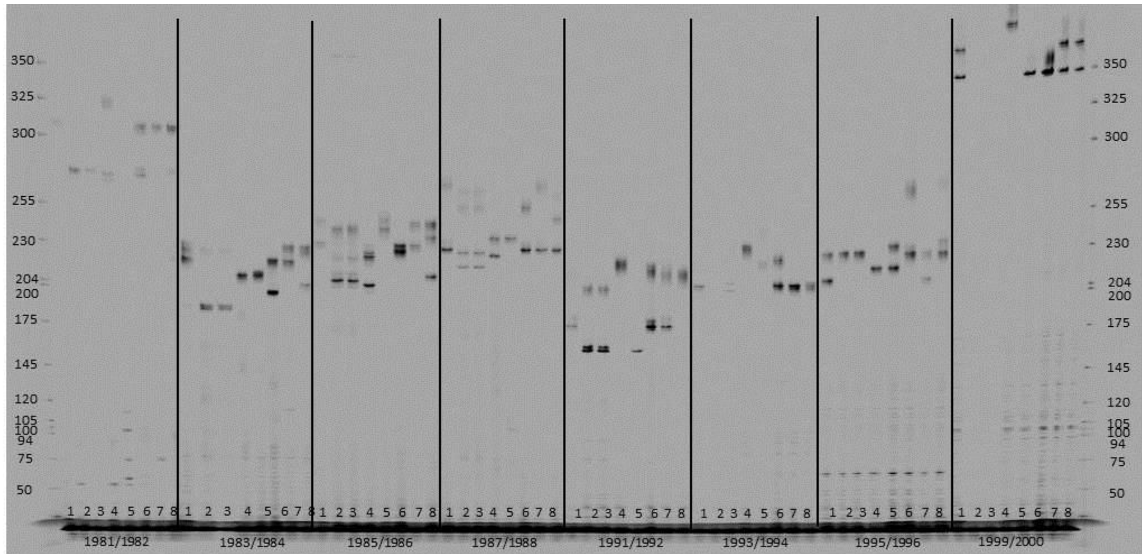
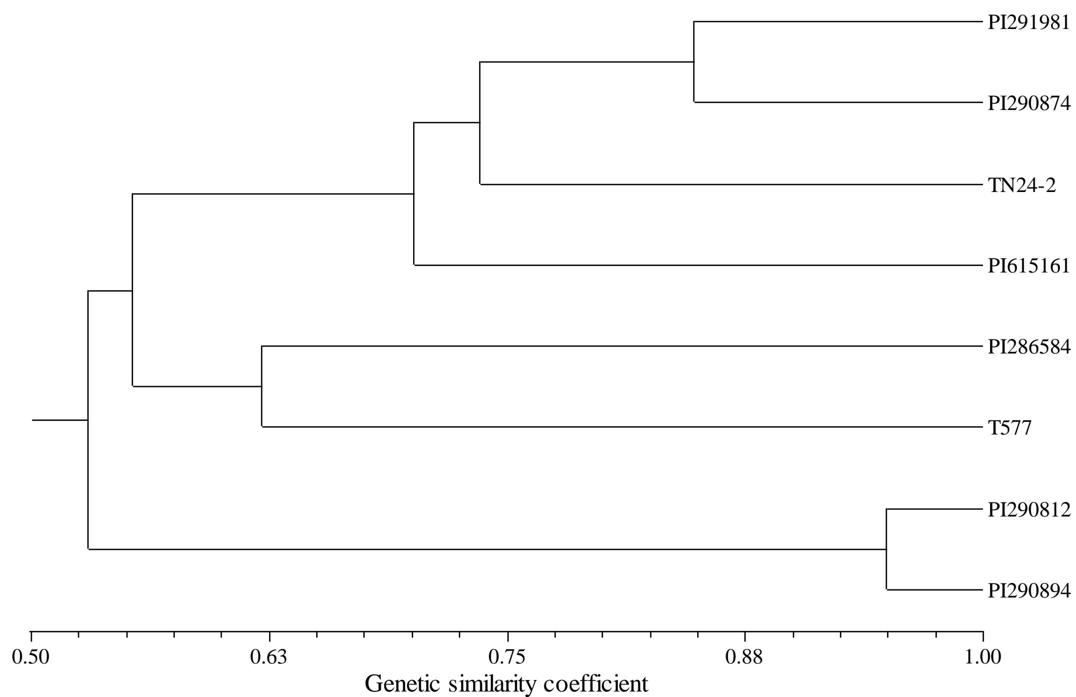


Figure 2.4. The tree plot constructed using unweighted pair group method with arithmetic average for eight *C. transvaalensis* genotypes based on 3971 amplified SSR bands.



CHAPTER III

SELFING AND OUTCROSSING FERTILITY IN *C. DACTYLON* VAR. *DACTYLON* (L.) PERS. UNDER OPEN-POLLINATING CONDITIONS EXAMINED BY SSR MARKERS

Abstract

C. dactylon var. *dactylon* (L.) Pers., common bermudagrass is a cosmopolitan grass species having great economic value for turf, forage and soil conservation in the southern United States. The species has long been characterized as an outcrossing species based on heterogeneity of natural populations, comparison of seed set rates under self- and cross-pollination, and assumption of widespread self-incompatibility. However, reproductive behavior of plants in breeding populations based on definitive characterization of their progeny as to self-or cross-pollinated origin is not available. The objective of this experiment was to quantify selfing and outcrossing rates of common bermudagrass plants grown in field environments conducive to interbreeding. Twenty five clonal common bermudagrass plants grown at Stillwater and Perkins, OK in a randomized complete block design with three replications were used in the study. Ten progeny from open-pollinated seed harvested from each field plot of the maternal plants were grown in a greenhouse at the Agronomy Research Station, Oklahoma State University. DNA samples were isolated respectively from 1439 progeny from both sites along with their 25 seed parents. Eleven polymorphic simple sequence repeat (SSR) markers were chosen to identify breeding origin of the progeny by comparing SSR marker genotypes of the progeny with their respective maternal parents. Among the progeny examined, only two progeny plants respectively.

from accessions A12281 and A12363 were identified to be selfed, indicating an extremely high outcrossing rate of 99.86%. The finding of complete or near complete outcrossing behavior in this experiment should assist in fuller understanding of the sexual reproduction characteristics of common bermudagrass.

INTRODUCTION

C. dactylon var. *dactylon* (L.) Pers., common bermudagrass, is a cosmopolitan grass species having great economic value for turf, forage and soil conservation in the southern United States (Harlan, 1970; Beard, 1973; Taliaferro, 2003). The predominant mode of sexual reproduction in *C. dactylon* has been characterized as outcrossing due to cross-pollination and self-incompatibility. Based on general observations of highly variable bermudagrass plants growing along a railroad track, Burton (1947) deduced the species might reproduce via outcrossing but did not attempt to determine rates of selfing vs. outcrossing. The wide distribution of self-incompatibility in the species and low percent seed set when self-pollinated have been reported by Kneebone (1967), Richardson et al. (1978) and Brede et al. (1989). Burton and Hart (1967) reported bermudagrass plants set six times more seed when crossed than selfed. Richardson et al. (1978) reported self-pollination seed set of bagged inflorescences of 30 common bermudagrass plants ranged from 0.10 to 8.09%, but self-fertility of 24 of the 30 selected plants was less than 1%. In their experiment, the open pollination seed set of the same plants varied from 15 to 57% (Richardson et al., 1978).

While comparison of seed set percentage under self- vs. open-pollination has value in predicting levels of pollination mode it does not distinguish plants emanating from open-pollinated seed as to self- vs. outcrossed origin. With the recent development of simple sequence repeat (SSR) markers, it is feasible to determine the fertilization mode in *C. dactylon*. As a co-dominant molecular marker system, SSR markers are a powerful tool for genetic diversity

analysis, linkage map construction, quantitative trait loci analysis, and marker-assisted selection in major crops, such as maize (Benchimol et al., 2005), rice (Ashkani et al., 2012), wheat (Zhou et al., 2008) and sugarcane (James et al., 2012). SSR markers analyze one locus at a time and readily identify homozygous and heterozygous genotypes. As a polymerase chain reaction (PCR) based technology, it requires a small amount of DNA as reaction templates and generates reliable polymorphic bands efficiently through the testing population. Thus, SSR marker technology has become an ideal molecular marker tool in bermudagrass regarding cultivar identification (Wang et al., 2010; Harris-Shultz et al., 2011), germplasm genetic diversity analysis (Ling et al., 2012; Wang et al., 2013) and genetic linkage mapping (Harris-Shultz et al., 2010). Transfer SSR and EST-SSR markers in bermudagrass have been developed and available in public (Harris-Shultz et al., 2010; Tan et al., 2012). A large set of *C. dactylon* genomic SSR markers were recently developed in our laboratory (manuscript in preparation).

Progeny array approach is commonly applied to estimate mating system parameters, by which the data are recorded from the difference of polymorphic markers between maternal plants and their respective half-sib progeny. To date, no quantitative information has been published on mating behavior of common bermudagrass in naturally pollinating environments. Thus, the objective of this study was to determine selfing and outcrossing rates of *C. dactylon* plants grown in the field using SSR markers.

MATERIALS AND METHODS

Plant materials and experimental design

Twenty-five Chinese *C. dactylon* clonal accessions having relatively high fertility were chosen from 'the Oklahoma State University turf bermudagrass germplasm nursery' (Table 1) (Wu et al., 2006a). All of the bermudagrass clones are tetraploid ($2n=4x=36$ somatic chromosomes) and sexually fertile (Wu et al., 2006b). In July 2011, field plots were established

in a randomized complete block design (RCBD) with three replications at the Agronomy Farm and Cimarron Valley Research Stations in Stillwater and near Perkins, OK, respectively. Plots were established by planting four single potted plants that were allowed to ultimately fill a 1.52 m $\times$ 2.44 m area. A 1.52 m alley was maintained between plots. The soil types present at the Stillwater Cow Creek research site and the Cimarron Valley Research Station were respectively an Easpur loam and a Teller fine sandy loam. Based on the soil test reports from the two locations, fertilizers [N-P(P_2O_5)-K(K_2O)] were applied at 56, 112 and 112 kg ha^{-1} respectively to both fields to achieve optimum fertility in bermudagrass before transplanting.

Field management

Immediately after the clonal plants transplanting into the field at the Cow Creek Bottom site of the OSU Agronomy Research Farm on 7 and 8 of July and at Cimarron Valley Research Station in Perkins on 22 of July, 2011, Dual® herbicide (metalochlor) was applied to both fields at 3.36 kg ha^{-1} a.i. Weeds between the alleys were suppressed by applying 2.24 kg ha^{-1} a.i. Roundup (glyphosate, N-phosphonomethyl glycine) plus surfactant (0.5% $v:v^{-1}$) and 2.24 kg ha^{-1} $(NH_4)_2SO_4$ herbicide in middle of September.

Seed harvesting, germination and tissue collection

In August 2012, twenty mature inflorescences were randomly hand harvested from each plot, resulting in 150 seed samples from the two field trials. Samples were collected into individual pre-labeled paper bags and kept about a week at room temperature for dehydration. Seedheads from each bag were threshed by rubbing in pans lined with ridged rubber matting and cleaned with a Model B South Dakota seed blower (Seedburo Equipment Co., IL). The cleaned seeds of the respective samples were planted in Metro Mix 250 growing medium (Sun Gro Horticulture, Bellevue, WA) in cells (4.875 inch $\times$ 7.75 inch for each insert) inside a white tray (10.5 inch $\times$ 21 inch) for germination in a greenhouse at the OSU Agronomy Research Station.

Ten randomly selected seedlings from each seed sample were respectively transplanted into conetainers. They were watered daily and fertilized periodically to provide healthy growth for leaf-tissue sampling. Twenty-five maternal plant leaf tissue samples were hand collected individually from field plots at the OSU Agronomy Farm in August 2012 and then kept in a -20 ℃ freezer for further DNA isolation.

DNA isolation and SSR markers selection

Genomic DNA was extracted from 0.15g frozen leaf tissue of each of the progenies and their parents using the CTAB method of Doyle and Doyle (1990). DNA concentrations were quantified using a NanoDrop DN-1000 Spectrophotometer (NanoDrop products, DE). Each DNA working solution was adjusted to a concentration of 10ng/μl as the template for polymerase chain reaction (PCR).

Twenty-five DNA testing panels were formed to perform SSR genotyping. Each panel consisted of 64 samples including four replications of one parent and its 60 progeny samples from two locations. Eleven SSR primer combinations (Table 2) were selected to genotype the maternal parents and their respective half-sib progenies. Five primer combinations (CDCA623-624, CDGA783-784, CDGA1795-1796, CDATG1999-2000 and CDAAC 2693-2694) were initially used to identify outcrossed and putative selfed progenies. After the initial screening, four new DNA panels were made to compose the remaining putative selfed progeny and their respective maternal parent DNA samples. The four panels were examined with six additional SSR primer pairs (CDCA55-56, CDCA77-78, CDCA155-156, CDCA379-380, CDCA491-492, and CDGA1179-1180). At the final step, the 11 SSR markers were combined in one testing panel to confirm identified selfed progeny.

PCR amplification

SSR-PCR amplifications were performed on Applied Biosystems 2720 thermal cyclers (Applied Biosystems Inc., CA) fitting in a 96-well PCR plate. Each reaction contained 4.3 µl nuclease free water, 1.0 µl 10×reaction buffer, 0.6 µl 25 mM $MgCl_2$, 0.2 µl 10 mM deoxynucleoside triphosphate (dNTP), 1.34 µl 1 pmol/µl forward primer, 1.34 µl 1 pmol/µl reverse primer, 0.2 µl 1 µM IR-M13 forward primer labeled with either 700- or 800-nm florescent dye (LI-COR, Lincoln, NE), 0.03 µl of 5U μl^{-1} AmpliTaq Gold DNA polymerase (Applied Biosystems) and 1.5 µl of 10 ng/µl template DNA. Thermal cycler parameters were programmed following Wu and Huang (2008). Five µl blue stop solution was added to each PCR reaction well, spun down, and denatured for 3 min at 94 ℃ in the 2720 thermal cycler (Applied Biosystems, IL). The PCR products from a plate labeled with 700 nm florescence dye and the other plate labeled with 800 nm florescence dye (LI-COR Inc., NE) were mixed together. The amplified PCR products were placed on ice until gel loading or stored at 4 ℃.

Gel electrophoresis and data analysis

To score each amplified target band the mixed products were loaded into wells of 6.5% KB^{plus} LI-COR gel (LI-COR Inc., NE) and run at 1500 volts for 1 hour and 45 min in a LI-COR 4300 DNA Analyzer (LI-COR Inc., NE). The 50-350bp standard size markers (LI-COR Biosciences, Lincoln, NE) were loaded into wells on both sides of the gel to determine the size of the amplified fragments. Bands in target regions were visually scored.

Progeny array approach was employed to identify the selfed progenies by comparing the open-pollinated progeny SSR genotypes to their respective maternal parents. Progeny showing one or more foreign bands were considered to have originated via outcrossing. Progeny not showing any foreign bands after analysis with the 11 SSR markers were considered to have originated via selfing. Microsoft Excel was used to record data and calculate the cumulative outcrossing and selfing rates in the 25 half-sib families.

RESULTS AND DISCUSSION

Half-sib progenies from seed germination

A total of 1439 half-sib progenies were used for the genotyping research. The missing 61 progenies resulted either from inadequate seed germination or from DNA isolation failure. The number of progenies from each of the 25 female parents tested ranged from 52 to 60 with an average of approximately 58 progeny per family (Table 3.3).

SSR markers selection and screening

In the initial screening, nine genomic SSR primer pairs were selected from a large SSR marker set developed in our laboratory (manuscript in preparation). They were chosen on the basis of PCR amplification quality and reliability (Figure 3.1). Two of the nine selected SSR markers were discarded later as either they produced ambiguous bands or amplification failure occurred through the testing panels. The other four SSR primer pairs were chosen from the previous study by Wang et al. (2010) based on their relatively high power of discrimination, which showed clear and strong amplification in our study as well.

Determination of percentages of crossed and selfed progeny

One progeny out of 1439 tested progenies was considered to have originated from a contaminant seed because none of its amplifications shared any maternal bands from the 11 SSR markers. The remaining 1438 progenies from 25 parents were tested as true progeny because at least one maternal band was present in the progeny amplification by each SSR marker. For all 25 half-sib families, the collective percentage of identified crossed progeny increased with the increasing number of SSR markers applied. The outcrossing rate of all families from three replications at each of the two locations started from 35.1% by one SSR marker, 65.4% by two, 80.8% by three, 90.5% by four, 96.5% by five, 98.8% by six, and 99.8% by seven to 99.9% by

eight SSR markers (Figure 3.2). Two progenies showed no foreign bands compared with the genotypes of their respective maternal parents, indicating they were putative selfed progeny after genotyping by 8 SSR markers. Data from three additional SSR markers genotyped on the two putative selfed progenies further indicated their self-pollination origin. After genotyping with the 11 SSR markers, only two of the 1438 progeny plants showed no foreign bands (Figures 3.3 & 3.4). Progeny PR1-35-1 was from Accession A12281 in the Cimarron Valley Research Station trial. Progeny SR3-125-10 was from Accession A12363 grown in the Agronomy Research Station.

Our results clearly demonstrated complete or near complete outcrossing to be the reproductive mode among the 25 common bermudagrass plants grown in field environments conducive to cross-pollination. Our experiment did not attempt to measure self-fertility of the 25 plants under enforced self-pollination. Previous research has demonstrated variation in self-fertility of common bermudagrass plants under enforced self-pollination (Richardson et al., 1978). In general, the reported self-fertility of self-pollinated common bermudagrass plants has been in the approximate range of 0 to 10%. 'Zebra', a common bermudagrass plant with chlorotic leaf stripes was reported to be highly self-fertile (Johnston and Taliaferro, 1975; Taliaferro and Lamle, 1997). The very high outcrossing in our experiment is likely highly influenced by greater competitive ability of pollen from other plants vs. self-pollination. A cytological study by Taliaferro and Lamle (1997) elegantly revealed significant differences in pollen tube growth post pollination between cross-fertilization and self-fertilization in *C. dactylon* plants. They observed that pollen tubes readily entered into stigmatic tissues post germination regardless of pollination mode. However, growth rate of pollen tubes in stylar tissues varied upon genotype and pollination mode. Except those of 'Zebra', a self-compatible genotype, most pollen tubes of genotypes did not reach the micropylar region within 24 hours post self-pollination. Conversely, pollen tubes of all genotypes reached the micropylar region within 2.5 hours post

cross-pollination. Evidently self-pollination resulted in slow growth of pollen tubes which seldom grew into the micropylar region of ovaries in comparison to pollen tubes emanating from cross-pollination.

In conclusion, we report the fertilization mode of common bermudagrass plants under open pollination in the field conditions was complete or near complete outcrossing. The results assure most progeny are hybrids in single or poly crosses, which are commonly used in the development of bermudagrass cultivars. The results add to the knowledge base regarding the sexually reproductive biology of common bermudagrass.

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Table 3.2. SSR marker ID, primer sequences, repeat motifs, and their predicted product sizes for genotyping 25 populations.

Table 3.3. The number of open pollinated seedlings from 25 maternal parents in two experimental locations for SSR analysis.

Table 3.1. Information of 25 *Cynodon dactylon* accessions from China with their accession identification (OK accession No., original Chinese No. in parentheses) and geographic origins.

No.	Identification	Origin	No.	Identification	Origin
1	A12269 (23)	Sichuan	14	A12321 (75)	Sichuan
2	A12274 (28)	Sichuan	15	A12330 (85)	Sichuan
3	A12279 (33)	Sichuan	16	A12342 (98)	Sichuan
4	A12280 (34)	Sichuan	17	A12345 (101)	Sichuan
5	A12281 (35)	Sichuan	18	A12347 (103)	Sichuan
6	A12283 (37)	Sichuan	19	A12350 (106)	Guangdong
7	A12288 (42)	Sichuan	20	A12356 (114)	Zhejiang
8	A12306 (60)	Sichuan	21	A12357 (116)	Jiangsu
9	A12309 (63)	Sichuan	22	A12358 (117)	Jiangsu
10	A12310 (64)	Sichuan	23	A12359 (118)	Jiangsu
11	A12311 (65)	Sichuan	24	A12361 (121)	Jiangsu
12	A12315 (69)	Shanghai	25	A12363 (125)	Jiangsu
13	A12318 (72)	Shanghai			

Table 3.2. SSR marker ID, primer sequences, repeat motifs, and their predicted product sizes for genotyping 25 populations.

No.	Marker ID	Primer sequences (5'-3')	Repeat motif	Predicted product size
1	CDCA 55-56	F:CGAGTCCATGCCTAACTCAA	(GT)9	322
		R:ACGGAAGGGTCAGTGGTAAC		
2	CDCA 77-78	F:GAAGATGTCATCACGATGGG	(GT)25	213
		R:CGTACGACCGAGTTCTCTGA		
3	CDCA 155-156	F:CTCCCTCGTCCATTTCATTT	(TC)16-(AC)15	290
		R:CGTTGGCACTCACTACCAGT		
4	CDCA 379-380	F:AGCACAGGCTTCTTATGCAA	(AC)7-(GT)37	318
		R:TTATGAAGATAGCCCGGTCC		
5	CDCA 491-492	F:CTTGGTTCTTGGGTCCTTGT	(GT)12	270
		R:AGCTCAAGCACCATTGTCAG		
6	CDCA 623-624	F:CGAGACCTAGTGAACAGCGA	(AC)17-(CA)5	310
		R:GGCCGTGCTTAAAGGAATAG		
7	CDGA 783-784	F:CACTGTTTACCCATCCAACG	(GA)14	221
		R:TTTTCGTACACACCCCAGAA		
8	CDGA 1179-1180	F:GGGCAGGACAGTTAACGAAT	(GA)9	202
		R:CTACCCAGCCAACCTGACTT		
9	CDGA 1795-1796	F:TTCGTGGACTCTGGCTATTG	(AC)5-(AG)36	345
		R:GCCCAGGTAACGTGTTCTTT		
10	CDATG 1999-2000	F:CCAGGTTCGCATCAGATA	(ATG)7	259
		R:TGCATATCATGAACACGACG		
11	CDAAC 2693-2694	F:TTGCCTACCAAACACGAAAG	(AAC)7	302
		R:TCCAAACTCGTGTAATTGCC		

Table 3.3. The number of open pollinated seedlings from 25 maternal parents in two experimental locations for SSR analysis.

Parent ID with Chinese No.	Stillwater	Perkins	Total
23	30	29	59
28	30	30	60
33	29	30	59
34	30	30	60
35	30	28	58
37	30	28	58
42	30	30	60
60	29	30	59
63	28	30	58
64	30	30	60
65	30	30	60
69	30	30	60
72	27	30	57
75	29	24	53
85	30	28	58
98	30	30	60
101	30	28	58
103	24	30	54
106	24	28	52
114	27	30	57
116	25	30	55
117	21	29	50
118	29	30	59
121	25	30	55
125	30	30	60
Total			1439

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Figure 3.1. Initial screening gel images of 60 progeny and their maternal parent DNA with 5 SSR markers. Each image was labeled with SSR marker ID and Chinese identification No. for population on the top left. Progeny samples were grouped by braces indicating the location and replication with their respective maternal parent DNA samples underlined in the middle and both sides of each gel. Standard size marker (bp) on the right side of each gel was given to locate the amplification in the testing panels.

Figure 3.2. Outcrossing percentages of progeny from twenty five populations examined with increasing the number of SSR markers from 1 to 8.

Figure 3.3. Gel image of progeny PR1-35-5 and its maternal parent examined by 11 SSR markers. SSR markers information was listed at the bottom to indicate individual genotyping. Within each individual genotyping, two duplicate progeny DNA samples were genotyped in the middle of each marker with one maternal parent DNA on each side. Standard size marker (bp) on the right side of gel image is given to locate the amplification in the testing panel.

Figure 3.4. Gel image of progeny SR3-125-10 and its maternal parent examined by 11 SSR markers. SSR markers information is listed at the bottom to indicate individual genotyping. Within each individual genotyping, two duplicate progeny DNA samples were genotyped in the middle of each marker with one maternal parent DNA on each side. Standard size marker (bp) on the right side of gel image is given to locate the amplification in the testing panel.

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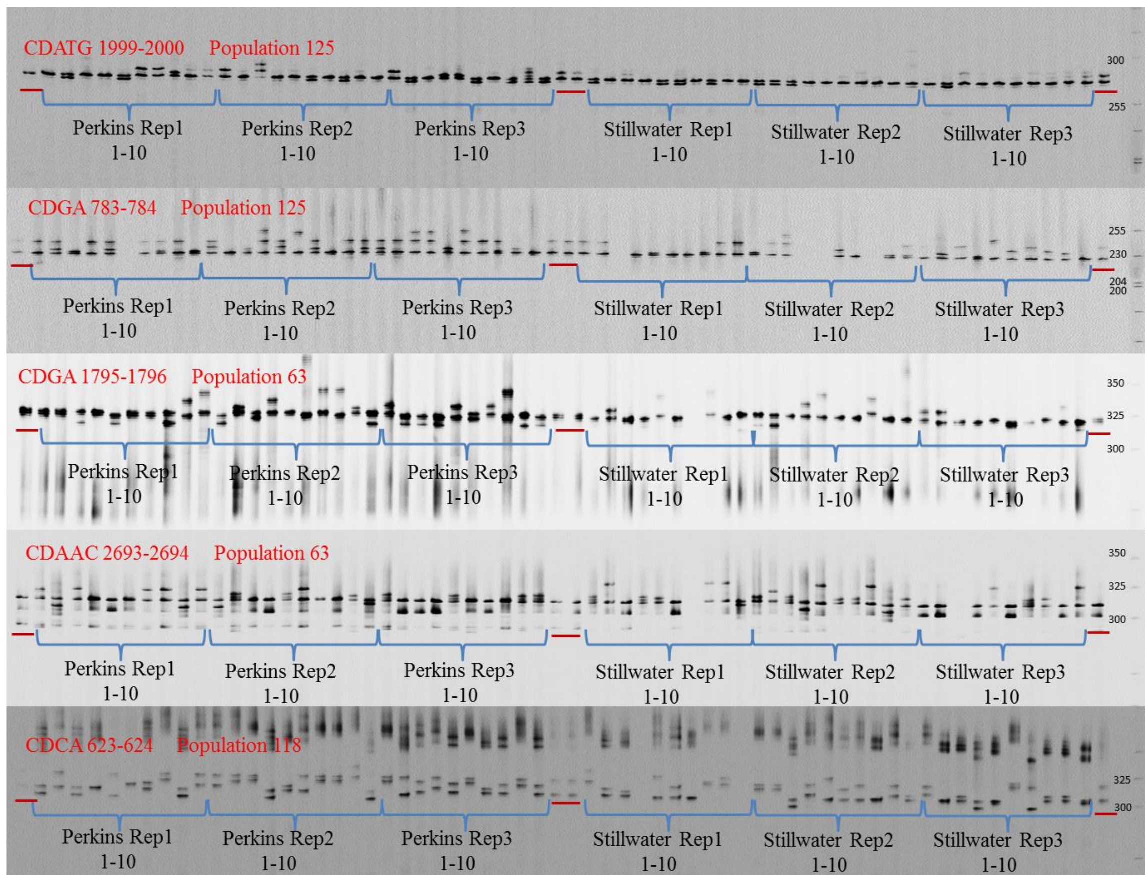


Figure 3.2. Outcrossing percentages of progeny from twenty five populations examined with increasing the number of SSR markers from 1 to 8.

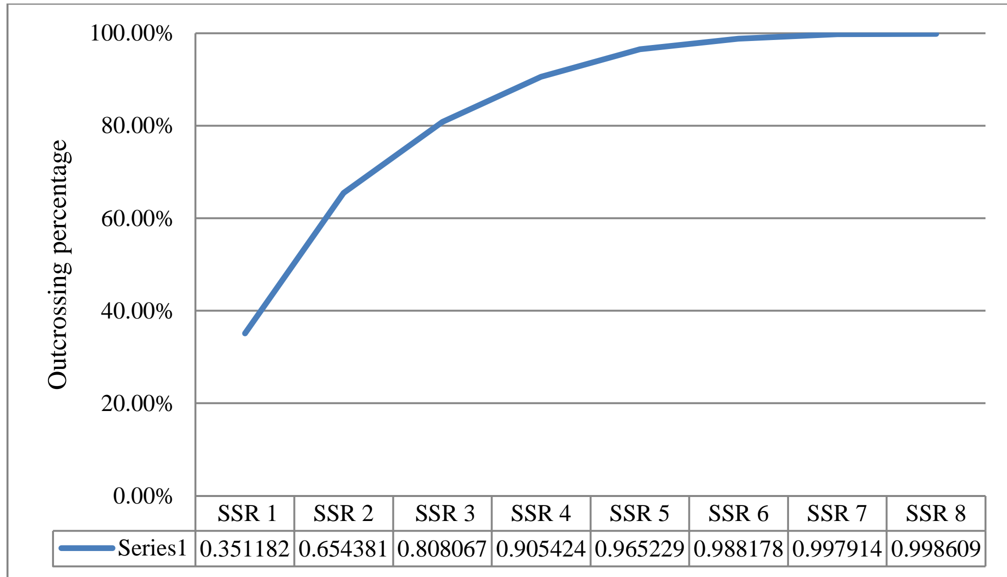


Figure 3.3. Gel image of progeny PR1-35-5 and its maternal parent examined by 11 SSR markers. SSR markers information is listed at the bottom to indicate individual genotyping. Within each individual genotyping, two duplicate progeny DNA samples were genotyped in the middle of each marker with one maternal parent DNA on each side. Standard size marker (bp) on the right side of gel image is given to locate the amplification in the testing panel.

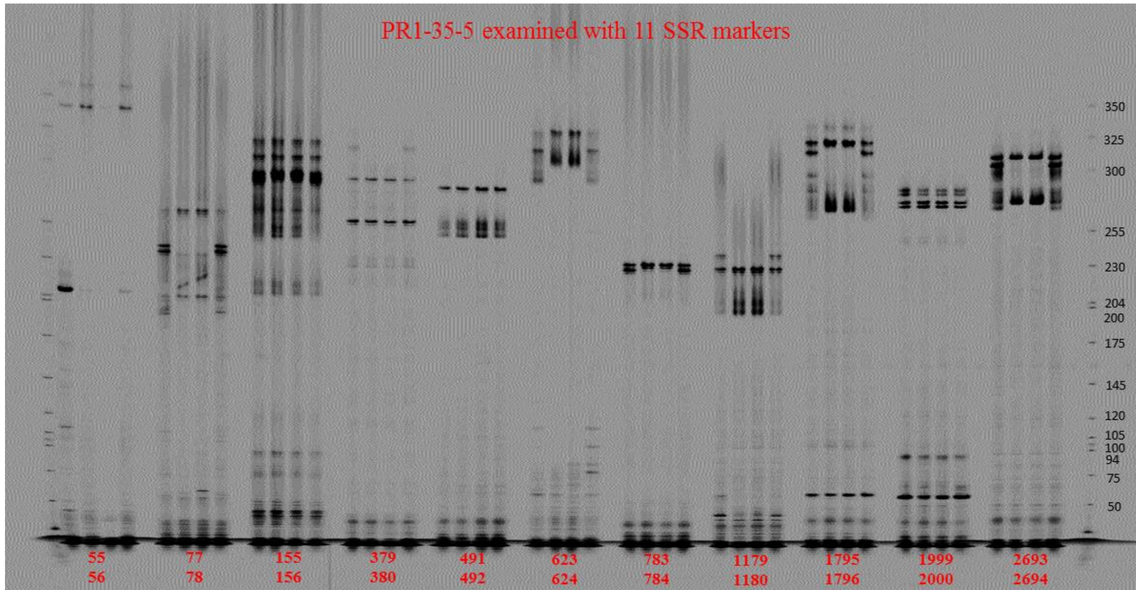
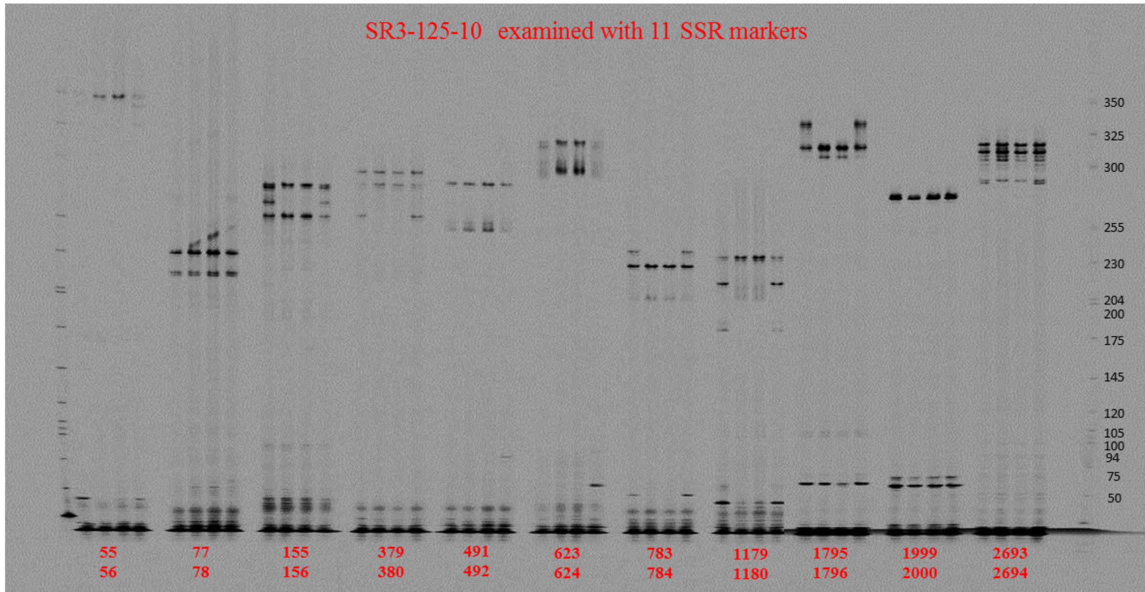


Figure 3.4. Gel image of progeny SR3-125-10 and its maternal parent examined by 11 SSR markers. SSR markers information is listed at the bottom to indicate individual genotyping. Within each individual genotyping, two duplicate progeny DNA samples were genotyped in the middle of each marker with one maternal parent DNA on each side. Standard size marker (bp) on the right side of gel image is given to locate the amplification in the testing panel.



CHAPTER IV

HERITABILITY ESTIMATES FOR SEED YIELD AND ITS COMPONENTS IN *CYNODON DACTYLON* VAR. *DACTYLON* (L.) PERS.

ABSTRACT

Seed yield is a major trait targeted for improvement in bermudagrass [*Cynodon dactylon* var. *dactylon* (L.) Pers.] breeding programs because of the increased interest in seed-propagated cultivars. Understanding the nature of genetic variation for seed yield and its components in bermudagrass would aid development of seed-propagated bermudagrass cultivars. The objective of this study was to estimate the genetic component of variation and narrow-sense heritability for seed yield and its two major components, inflorescences prolificacy and seed set percentage in bermudagrass. Twenty-five half-sib families and their respective clonal parents were evaluated at two Oklahoma locations, Perkins and Stillwater in 2012 and 2013. Half-sib families were different for seed yield, inflorescences prolificacy and seed set percentage, indicating the expression of additive genes in controlling these traits. Family × location effects were observed for seed set percentage and seed yield. All three traits showed family × year interaction effects. There was a significant family × location × year interaction in inflorescences prolificacy and seed set percentage. Narrow-sense heritability estimates for seed yield was 0.18 based on variance component analysis among half-sib families and ranged from 0.26 to 0.68 based on parent-offspring regressions, indicating a complex genetics of seed yield. Heritability estimates were moderate (0.30-0.55) for inflorescences prolificacy and moderate to relatively high (0.41-0.78)

for seed set percentage. These results indicate that sufficient magnitudes of additive genetic variation for seed set percentage and inflorescence prolificacy permit positive response to selection and conventional progeny-based genotypic evaluation is necessary for seed yield improvement.

INTRODUCTION

Bermudagrass [*Cynodon dactylon* var. *dactylon* (L.) Pers.] is economically the most important and genetically most diverse species in the genus *Cynodon* Rich. It has been widely used for turf, forage, soil conversation and remediation of contaminated soil in southern United States and other temperate and tropical regions of the world (Beard 1973; Taliaferro 2003). Bermudagrass has long been characterized as an outcrossing species.

Sexual reproduction via cross-pollination and self-incompatibility is responsible for the immense genetic and phenotypic variability among bermudagrass (Burton, 1947; Burton and Hart, 1967; Taliaferro and Lamle, 1997). The broad genetic diversity within the species has been primarily derived from combinational functions of sexual reproduction and natural selection during its dispersion and subsequent adaption around the world. The sexual reproduction capability of individual bermudagrass plants varies from none to very high (Harlan and de Wet, 1969). In general, seed production of bermudagrass is low, but most plants have the ability to produce some seed that effects genetic recombination and segregation. According to Harlan and de Wet (1969), the variability within *C. dactylon* for many characteristics, including fertility, was enormous and generated by population fragmentation based on chromosomal changes such as translocations and deletions. As one of the most prominent causes of the variation in seed yield in bermudagrass, the chromosomal irregularities are sufficient to cause high, but usually not complete sterility.

Extensive variability among selected genotypes of bermudagrass for components of seed yield has been documented (Ahring et al., 1974; Burton, 1947; Burton, 1951; Richardson et al., 1978). Burton (1947) studied variation in many characteristics of 147 bermudagrass polycross progenies, including seedhead abundance, seed set and seed yield. He concluded that a considerable amount of variation existed among polycross progenies for seedhead abundance, seed set percentage and seed yield. Seed yield was correlated (r=0.59) with seed set percentage. Ahring et al. (1974) collected data from seed yield and its components in bermudagrass using seven single-crosses with seven male clones and one cultivar as maternal parent. The components included number of racemes per head, number of florets per raceme, number of florets per head and number of florets containing a caryopsis per head (seed set percentage). Significant differences were found between progenies and parents for all components except florets per head. Paternal parentage could affect the seed set, because the differences in progenies were a result of different male clones. Seed yield of any offspring did not exceed that of the highest yielding parents.

Wu et al. (2006) reported an enormous amount of genetic variability and relationships for seed yield and its components existing in Chinese tetraploid *C. dactylon* accessions. Using path coefficient analyses, they concluded that inflorescence prolificacy (r=0.51) and seed set percentage (r=0.38) were significantly positively correlated with seed yield and had the highest direct effects on seed yield, indicating that selection for increased inflorescence prolificacy and seed set should be the best indirect method for the improvement of seed yield.

Since the 1980s, the number of seed-propagated turf bermudagrass cultivars released for commercial production has dramatically increased, especially in the breeding programs belonging to commercial companies in the USA (Taliaferro, 2003). According to Ahring et al. (1974), seed-propagated bermudagrass cultivars are needed because commercial planting equipment and establishment technology for vegetative propagation are not effective for confined areas, such as

home lawns or on steep embankments such as dam faces or roadsides. In addition, vegetative propagation requires more time and physical labor than seeding. Establishment of bermudagrass turf is less expensive by seeding than by sodding (Patton, 2012). With the increased interest in breeding seed-propagated bermudagrass cultivars, seed yield has become a major trait targeted by bermudagrass breeding programs.

Heritability is a measure of the magnitude to which genetic factors contribute to the quantitative phenotypic variation in a population. In breeding programs, information regarding heritability is most useful as a measure of potential response to selection, i.e. predicted genetic gains per selection cycle. Broad sense heritability (H^2) measures the magnitude to which phenotypic variance (V_p) is determined by variation in genetic factors (V_g) of additive, dominant and interactive effects. Narrow sense heritability (h^2) only quantifies the proportion of phenotypic variation that is due to additive genetic effects.

Robust information on narrow sense heritability estimates for seed yield and its components is warranted to increase the selection efficiency for such traits during the development of improved seed-propagated bermudagrass cultivars. Accordingly, the objectives of this study were (i) to determine if significant genetic variability exists for seed yield and its related components among selected half-sib families of *C. dactylon* accessions, (ii) to calculate narrow-sense heritability estimates for seed yield and its two major components using variance components analysis and parent-offspring regression, and (iii) to use this information to suggest appropriate breeding strategies for the development of seed-propagated bermudagrass cultivars.

MATERIALS AND METHODS

Plant materials

Plant materials used in the study included 25 *Cynodon dactylon* clonal accessions introduced from China and half-sib progeny from the accessions. The 25 clonal accessions were

selected from a larger population of accessions from China based on their winter hardiness and relatively high fertility (Table 4.1). Half-sib seed from the 25 respective clonal accessions was harvested from plots in a replicated nursery in 2002 (Wu et al., 2006).

About 0.1g (~2000 seeds/g) seed of each half-sib family was planted in Metro Mix 250 growing medium (Sun Gro Horticulture, Bellevue, WA) in labeled 12.4 cm by 19.7 cm black pots placed inside a 26.7 cm by 53.3 cm white tray in a greenhouse at the Agronomy Research Station, Oklahoma State University (OSU). Forty-eight progeny seedlings from each pot were transplanted to two 24-cell trays (26.7 cm $\times$ 53.3 cm) representing one half-sib family. As the experiment was planned for two locations, two sets of the same progeny seedlings per family were prepared. After a 2-3 month growing period, 40 plants in each of two containers were randomly selected and respectively split into three clones which were used in establishing experiments on the OSU Agronomy Research Station, Stillwater, OK (Table 4.2) and the Cimarron Valley Research Station, Perkins, OK (Table 4.3). Propagating material for each of the 25 maternal parent plants was dug from the '2007 OSU turf bermudagrass germplasm nursery'. Six identical potted plants were vegetatively prepared for each of the 25 parents in a greenhouse at the OSU Agronomy Research Station, Stillwater.

Experimental design

The experimental design for the Stillwater and Perkins experiments was a randomized complete block with three replications. Within each replication, 25 randomly selected half-sib families and their respective 25 maternal clones were randomly arranged and planted into each 1.52 m $\times$ 2.44 m (5 feet $\times$ 8 feet) plot with 1.52 m (5 feet) bare borders between neighboring plots. Each of 40 progeny plants per family was planted on 30.5-centimeter (one foot) centers in each progeny plot. Four clonal plants of one maternal parent were transplanted to its respective

parent plot. The designs for establishing offspring and parents within each plot are shown in Figures 4.1 and 4.2.

The plot plans of bermudagrass accessions with their original Chinese No. ('P' representing parent plants, 'O' as offspring plants) for field establishment in Stillwater and Perkins are given in Table 4.2 and Table 4.3, respectively.

Management procedures

The soil type at the Stillwater research site is an Easpur loam while the soil type at the Cimarron Valley Research Station is a Teller fine sandy loam (Natural Resources Conservation Service at http://websoilsurvey.nrcs.usda.gov). Based on the soil test reports for the two locations, fertilizers [N-P(P_2O_5)-K(K_2O)] were applied at 56, 112 and 112 kg ha^{-1} respectively to both fields to achieve optimum rates for bermudagrass seed production before transplanting. The Stillwater and Perkins experiments were respectively established July 7-8 and July 22, 2011. Dual® herbicide (metalochlor) was applied to both fields at 3.36 kg ha^{-1} a.i. Weeds within the alleys were suppressed by applying 2.24 kg ha^{-1} a.i. Roundup® (glyphosate, N-phosphonomethyl glycine) plus surfactant (0.5% v:v^{-1}) and 2.24 kg ha^{-1} $(NH_4)_2SO_4$ herbicide in middle of September.

In 2012 there was no mowing of plant residues before initiation of spring regrowth due to early spring green up following the establishment. In 2013 early March, plant residues in the plots were mowed off at a 5.08 cm height. Before the initiation of bermudagrass spring green up, Roundup® (glyphosate, N-phosphonomethyl glycine) at 4.68 L a.i. ha^{-1}, 2,4-D [(2,4-dichlorophenoxy) acetic acid] at 1.17 L a.i. ha^{-1} and Barricade® (prodiamine) at 2.58 kg a.i. ha^{-1} with 1.17 L ha^{-1} of surfactant were applied in early March according to the labels of each herbicide. In early May for both years of 2012 and 2013, nitrogen fertilizer was applied at a rate of 67 kg ha^{-1} for both plots in Stillwater and Perkins. During the growing season, glyphosate tank

mixed with a surfactant and ammonium sulfate was applied to control weeds in alleys if needed on a weekly basis.

Data collection

Data were collected in August and September in both 2012 and 2013. Measured and visually rated response variables were 1) inflorescence prolificacy, 2) seed set percentage [total seed number per inflorescence and collective raceme length (mm) per inflorescence], 3) seed yield (kg ha^{-1}).

Inflorescence prolificacy were visually assessed for each plot with a rating scale from 1 to 9, with 1 indicating no inflorescences and 9 most abundant inflorescences. This variable was taken at the beginning of August in 2012 and early September in 2013 due to the difference at inflorescence maturity. To obtain seed set percentage on the plot mean basis, eighty mature inflorescences were hand-picked from each offspring plot and stored into pre-labeled paper bags. Two inflorescences were randomly selected from forty 30.5-centimeter (one foot) centers within the offspring plot using a 30.5 cm $\times$ 30.5 cm (one square foot) grid, where originally the 40 individual progeny were planted. For the parent plots, 20 individual inflorescences collected to obtain this variable. For achieving seed set for each plot, ten inflorescences per replication of each entry were randomly chosen to determine seed number and raceme length (mm) per inflorescence, which were used to calculate seed set percentage. Seed set percentage was calculated as: (number of caryopses $inflorescence^{-1}$/ number of spikelets $inflorescence^{-1}$)*100. The number of spikelets $inflorescence^{-1}$ was estimated with a linear formula: Y=8.4 + 0.79X (r^2 = 0.68, p<0.01) (Wu et al., 2006). The number of caryopses $inflorescence^{-1}$ was counted by soaking the seedhead samples in a 20% (v/v) bleach solution then examined the seedhead under a dissecting microscope at 10$\times$ magnification (Wu et al., 2006). For seed yield data collection in September 2012, the variable was estimated by harvesting all biomass of each progeny plot using

a sickle-bar mower. All harvested biomass was bagged, dried thoroughly and then threshed by hammermilling at 800 rpm using a 0.371 cm round hole screen (Ahring et al., 1982). Due to incomplete coverage for some parent plots in 2012, the biomass from 30.5 cm $\times$ 30.5 cm fully covered area was randomly selected and hand-clipped within each plot. The parent plot samples were threshed by rubbing in pans lined with ridged rubber matting. In 2013, all parent and progeny plots were harvested by a sickle-bar mower to obtain the seed yield for each plot. All bagged biomass samples were threshed following the same procedure as in 2012. All threshed samples from both parent and progeny plots were cleaned into pure seed with a Model B South Dakota seed blower using an air-valve setting of 15 °(Ahring et al., 1982).

Statistical analyses

Data were analyzed using the MIXED procedure of SAS version 9.3 (SAS Institute) to obtain estimates of variance components and GLM procedure to obtain mean squares and significance for each of various sources of variation. Estimates of narrow-sense heritability were computed for all three variables, inflorescence prolificacy, seed set percentage and seed yield. Narrow-sense heritability was estimated for the three variables based on the genetic components of variation among half-sib families. In this experiment, the genetic variance of half-sib families predominantly measured the additive genetic variation in the population. Estimates of narrow-sense heritability were obtained for two individual years 2012 and 2013 and for both years combined. For single year data analysis, the estimates of variance components of half-sib families were based on combined data at two locations. The half-sib families data were collected on the plot mean basis, the narrow-sense heritability on a phenotypic variance among half-sib family mean basis averaging over replications, years, and locations can be estimated as $h_{PFM}^2=\sigma_F^2/(\sigma_F^2 + \sigma_{FL}^2/l + \sigma_{FY}^2/y + \sigma_{FLY}^2/ly + \sigma_\gamma^2/rl + \sigma_\varepsilon^2/ryl)$ (Nguyen and Sleper, 1983). The sources of variation pertinent to variance components of interest, and expected mean squares for a random model are given in Table 4.4. For single year analysis, the sources of variation were the

first three in Table 4.4 for the data collected over locations. In this case, the estimates of narrow-sense heritability on a phenotypic mean basis is $h_{PFM}^2=\sigma_F^2/(\sigma_F^2 + \sigma_{FL}^2/l+ \sigma_\gamma^2/rl)$ (Nguyen and Sleper, 1983).

Parent-offspring regression was the other method used for the estimates of narrow sense heritability. Regression of progeny means on parental means evaluated under different environments can remove the potential bias due to non-genetic covariance between parent and offspring. The estimate in this case would be free of genotype × environmental interaction effect. In our study, the parent offspring regression was performed with parent and offspring data from different locations for the single year data to reduce upward bias caused by genotype × environment interactions (Casler, 1983). The estimates of h_n^2 were calculated by the following formula: $h_n^2 = 2 \times \beta_1$, where β_1 = the slope of the parent offspring regression (Falconer, 1989).

Predicted genetic gain was calculated for seed yield and its two components using the formula: $\Delta G = ck\, h_{PFM}^2\, \sigma_{PFM}$, where c represents parental control factor, k represents the standardized selection differential, h_{PFM}^2 and σ_{PFM} represent heritability and phenotypic standard deviations on a phenotypic mean basis (Nguyen and Sleper, 1983). Parental control factor c= 2 in this experiment, ecause superior parents are selected based on the mean performance of their half-sib progenies and intermated in isolation to produce the improved population. For a selection intensity of 30%, k = 0.736 (Falconer, 1989).

RESULTS AND DISCUSSION

Weather conditions

Severe drought prevailed during the summer of 2012 while the 2013 summer received consistent rainfall during May, June and July (Figure 4.3). The monthly precipitation at the Stillwater site was 2.84, 5.49 and 0.18 cm in May, June and July of 2012 respectively, while 15.80, 10.03 and 14.15 cm rainfall occurred in the respective months of 2013. At the Perkins site

the monthly rainfall was 2.84, 7.39 and 0.66 cm in May, June and July of 2012, compared to 17.81, 10.52 and 15.42 cm precipitation in the respective months of 2013. It has been documented that too much rainfall during these months can be detrimental to seed production (Ahring et al., 1982a). The average air temperatures during May (23.06 ℃, 23 ℃), June (26.22 ℃, 26.22 ℃) and July (30.83 ℃, 30.89 ℃) 2012 at Perkins and Stillwater respectively were similar. In 2013, the average air temperatures during May (18.89 ℃, 19.06 ℃), June (25.44 ℃, 25.56 ℃) and July (26.28 ℃, 26.44 ℃) at Perkins and Stillwater respectively were averagely lower than those in 2012.

Phenotypic variation

Means, standard errors and ranges of seed yield and its components in parents and progeny half-sib families are given in Table 4.5. For inflorescence prolificacy rating, the ranges of offspring observations fell within the ranges of the parents from either single years or two year data combined. Considering seed set percentage, only the range of offspring at the Stillwater site in 2013 fell within the range of the parents. The ranges of offspring for seed yield fell within the ranges of the parents except the data at the Perkins site in 2013. The means of offspring for seed set percentage and seed yield were mostly greater than the means of parents at the same site in same years. The ranges and means for these two variables indicate that transgressive segregation may exist in the offspring population.

Estimated components of variation

Based on the data of offspring in 2012 (Table 4.6), half-sib families differed greatly in all the variables measured, indicating a significant role of additive genes in seed yield and its related components of bermudagrass. The fixed effect of location was only significant for seed yield. Seed set percentage showed a significant family $\times$ location interaction. The effects of replication nested within location were observed for both seed set percentage and seed yield. In 2013, half-

sib families were also different for all the variables measured (Table 4.7). The fixed location effects were shown for inflorescence prolificacy and seed yield. Seed set percentage and seed yield were also influenced by family × location interaction. In both single-year analyses, a significant residual variance existed in all three variables, which may suggest that the phenotypic variation involving some genetic variance associated with among individuals of the same half-sib families within plots. In order to exploit the within family genetic variance, individual data within a family is required, but were not collected in this experiment.

In the two-year combined analysis (Table 4.8), the year effect was of greater magnitude than the effects due to location or location × year interaction for inflorescence prolificacy, seed set percentage and seed yield. The year effect was likely related to the uncommon rainfall that occurred in 2013. The results also demonstrated a significant amount of variation among half-sib families for inflorescence prolificacy and seed set percentage, suggesting a significant contribution of additive genes in these two components associated with the seed production. For seed yield, however, the expected means square due to half-sib families did not exceed the expected mean square due to family × year interaction. This could indicate greater genotype × environment interaction in controlling seed yield variation than its two components. The family × year interaction effect was likely due to different responses of the families to the obvious climate difference between the two years. On average, the seed yield in 2013 was a tenth to fifteenth of the yield in 2012 (Table 4.5). This significant year effect is likely caused by the unusually high precipitation during the seed production season (May, June and July) in 2013 compared to 2012 with intermittent rainfall under drought conditions. Growth-stress cycles with alternate wet and dry conditions are needed to stimulate bermudagrass seed production and flowering during May, June and July, which afterwards could produce a good seed crop in August (Ahring et al., 1982a). In 2013, the weather provided consistent rather than intermittent precipitation during the seed production season.

The significant genetic variances in the half-sib families for inflorescences prolificacy and seed set percentage indicated that additive genetic components account for these two traits comparing with small interaction variances. Thus, breeding strategies commonly used for bermudagrass improvement, such as recurrent phenotypic selection, should be effective in improving these two seed yield related components.

Estimates of narrow sense heritability

From the single year data, narrow-sense heritability estimates based on half-sib families' variance components for inflorescence prolificacy, seed set percentage and seed yield in 2012 were 0.70, 0.61 and 0.84 respectively, while in 2013 were 0.50, 0.63 and 0.23 respectively (Table 4.9). A large decline occurred in the seed yield estimates between 2012 and 2013. In 2013, when unusually consistent precipitation was received and no growth-stress cycles occurred during the seed production season, the seed yields of families at two locations were significantly affected compared to the relatively drought conditions in 2012. Greater family $\times$ location effect was shown in both 2013 than 2012, which could be accounted for by differences in precipitation pattern. Some families at Perkins may have been influenced more severely than those at Stillwater due to the difference between soil types at the two locations. In the combined year analysis, the narrow-sense heritability estimates were 0.55, 0.78 and 0.18, respectively, for inflorescence prolificacy, seed set percentage and seed yield. Seed set percentage showed relatively high heritability estimates with single year and year combined analysis. Low narrow-sense heritability estimates for seed yield in this study were comparatively lower than the result (h^2=0.42) reported by Cluff and Baltensperger (1991). This could be attributed to the large family $\times$ year interaction and residuals associated with random effects from replications nested within location, including sampling error. Low heritability estimates may be the result of large phenotypic variability caused by differing microenvironments and are indicated by a large residual variance term.

The heritability estimates based on parent-offspring regression for all variables are shown in Table 4.10. The regressions were only performed with half-sib families and parents from different locations in the same year to exclude the family $\times$ location interaction effect and the significant amount of year effect in reverse year analysis. The heritability estimates were statistically different from zero ($p<0.05$) for inflorescence prolificacy, seed set percentage and seed yield in 2012 when regressing half-sib families performance at one location on parents at another location, except the estimate for seed set percentage obtained by regression with half-sib families at Perkins site. In 2013, only estimates for seed set percentage were statistically different from zero. The estimates based on parent-offspring regressions for inflorescence prolificacy ranged from 0.30-0.53, and the estimates for seed set percentage ranged from 0.41-0.76, which were fairly comparable with the estimates for both traits obtained from variation among half-sib families. This may suggest the low effect of non-additive gene actions in influencing inflorescence prolificacy and seed set. The heritability estimates for seed yield ranged from 0.26-0.68, which were only statistically significant in 2012. The heritability estimates for seed yield based on parent-progeny regressions in 2012 were from 0.26 to 0.68 while 2013 heritability values were not different from zero. The inconsistent heritability estimates for seed yield by years and in two methods indicate the genetic complexity of the trait.

CONCLUSIONS

The population of 25 half-sib families used in the current study was derived from open pollination in a field planting in a randomized complete block design with three replications. The families were selected because of relatively high fertility, winter survivability and spring green-up of their maternal parents. Relatively high to moderate narrow-sense heritability estimates for inflorescence prolificacy and seed set percentage in this population indicated not only that a

substantial genetic variation existed in the two traits, but also the variability was significantly controlled by additive gene action in nature and therefore of value to breeders. The results suggest that a significant improvement for seed yield could be possible when applying phenotypic selection to the two components. Low and unstable heritability estimates for seed yield indicated the sole phenotypic recurrent selection when obtaining seed yield directly may not be very effective to improve the trait in bermudagrass. Conventional genotypic selection procedures are necessary to achieve improvement for seed yield increase.

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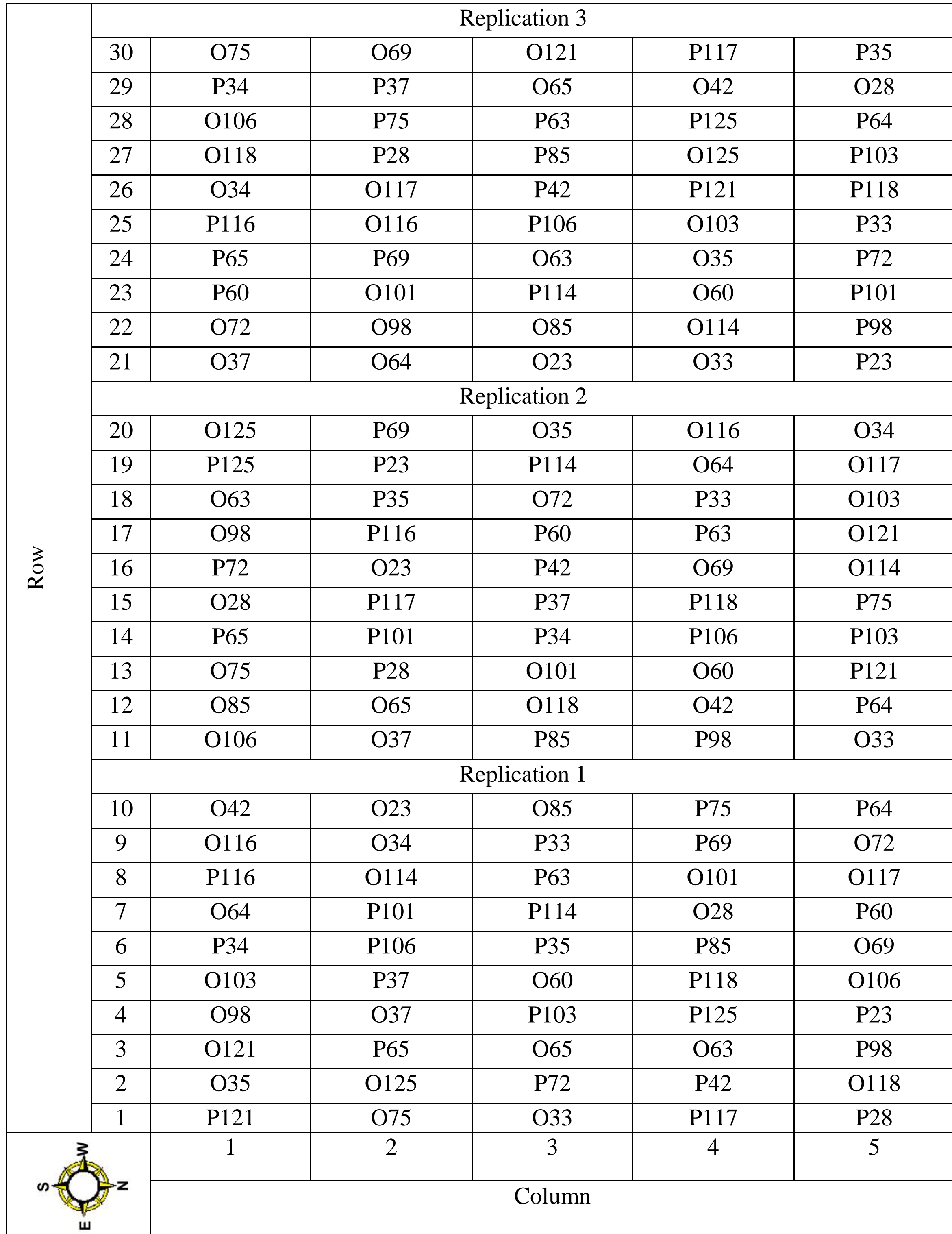
Table 4.1. Information of 25 *Cynodon dactylon* accessions from China with their accession identification (OK accession No., original Chinese No. in parentheses) and geographic origins.

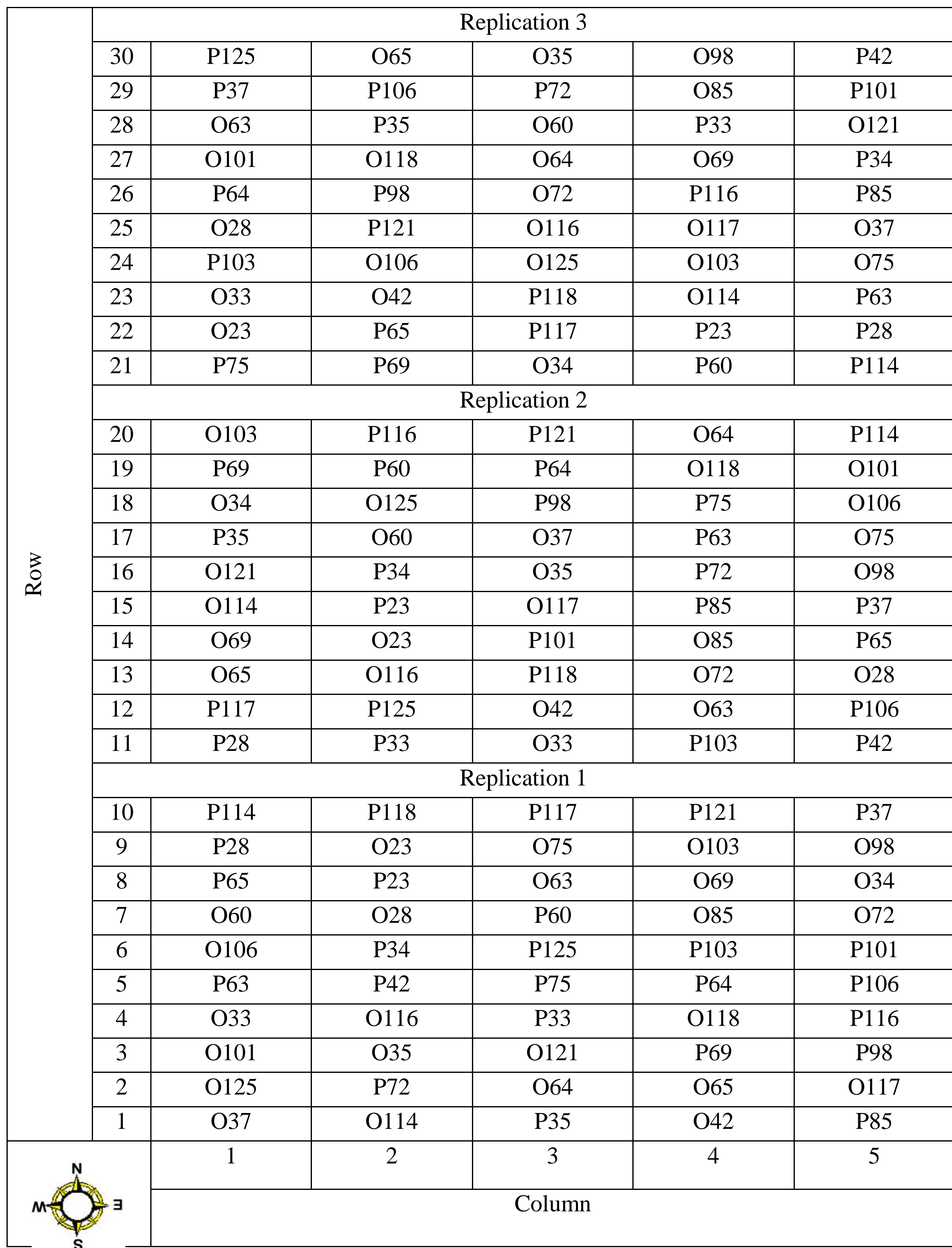
No.	Identification	Origin	No.	Identification	Origin
1	A12269 (23)	Sichuan	14	A12321 (75)	Sichuan
2	A12274 (28)	Sichuan	15	A12330 (85)	Sichuan
3	A12279 (33)	Sichuan	16	A12342 (98)	Sichuan
4	A12280 (34)	Sichuan	17	A12345 (101)	Sichuan
5	A12281 (35)	Sichuan	18	A12347 (103)	Sichuan
6	A12283 (37)	Sichuan	19	A12350 (106)	Guangdong
7	A12288 (42)	Sichuan	20	A12356 (114)	Zhejiang
8	A12306 (60)	Sichuan	21	A12357 (116)	Jiangsu
9	A12309 (63)	Sichuan	22	A12358 (117)	Jiangsu
10	A12310 (64)	Sichuan	23	A12359 (118)	Jiangsu
11	A12311 (65)	Sichuan	24	A12361 (121)	Jiangsu
12	A12315 (69)	Shanghai	25	A12363 (125)	Jiangsu
13	A12318 (72)	Shanghai			

Table 4.2. The plot plan of bermudagrass accessions with their original Chinese accession numbers established at OSU Agronomy Farm in Stillwater. ‘P’ represents parent, while ‘O’ indicates offspring.



Row						
	Replication 3					
	30	O75	O69	O121	P117	P35
	29	P34	P37	O65	O42	O28
	28	O106	P75	P63	P125	P64
	27	O118	P28	P85	O125	P103
	26	O34	O117	P42	P121	P118
	25	P116	O116	P106	O103	P33
	24	P65	P69	O63	O35	P72
	23	P60	O101	P114	O60	P101
	22	O72	O98	O85	O114	P98
	21	O37	O64	O23	O33	P23
	Replication 2					
	20	O125	P69	O35	O116	O34
	19	P125	P23	P114	O64	O117
	18	O63	P35	O72	P33	O103
	17	O98	P116	P60	P63	O121
	16	P72	O23	P42	O69	O114
	15	O28	P117	P37	P118	P75
	14	P65	P101	P34	P106	P103
	13	O75	P28	O101	O60	P121
	12	O85	O65	O118	O42	P64
	11	O106	O37	P85	P98	O33
	Replication 1					
	10	O42	O23	O85	P75	P64
	9	O116	O34	P33	P69	O72
	8	P116	O114	P63	O101	O117
	7	O64	P101	P114	O28	P60
	6	P34	P106	P35	P85	O69
	5	O103	P37	O60	P118	O106
	4	O98	O37	P103	P125	P23
	3	O121	P65	O65	O63	P98
	2	O35	O125	P72	P42	O118
	1	P121	O75	O33	P117	P28
		1	2	3	4	5
		Column				

Table 4.3. The plot plan of bermudagrass accessions with their original Chinese accession numbers established at Cimarron Valley Research Station in Perkins. ‘P’ represents parent, while ‘O’ indicates offspring.



Row	Column 1	Column 2	Column 3	Column 4	Column 5
	Replication 3				
30	P125	O65	O35	O98	P42
29	P37	P106	P72	O85	P101
28	O63	P35	O60	P33	O121
27	O101	O118	O64	O69	P34
26	P64	P98	O72	P116	P85
25	O28	P121	O116	O117	O37
24	P103	O106	O125	O103	O75
23	O33	O42	P118	O114	P63
22	O23	P65	P117	P23	P28
21	P75	P69	O34	P60	P114
	Replication 2				
20	O103	P116	P121	O64	P114
19	P69	P60	P64	O118	O101
18	O34	O125	P98	P75	O106
17	P35	O60	O37	P63	O75
16	O121	P34	O35	P72	O98
15	O114	P23	O117	P85	P37
14	O69	O23	P101	O85	P65
13	O65	O116	P118	O72	O28
12	P117	P125	O42	O63	P106
11	P28	P33	O33	P103	P42
	Replication 1				
10	P114	P118	P117	P121	P37
9	P28	O23	O75	O103	O98
8	P65	P23	O63	O69	O34
7	O60	O28	P60	O85	O72
6	O106	P34	P125	P103	P101
5	P63	P42	P75	P64	P106
4	O33	O116	P33	O118	P116
3	O101	O35	O121	P69	P98
2	O125	P72	O64	O65	O117
1	O37	O114	P35	O42	P85

Table 4.4. Analysis of variance on a plot mean basis for data collected over locations and years.

Source of variation	df	Expected mean squares
Families	(n-1)	$\sigma_{\varepsilon}^2+y\sigma_{\gamma}^2+r\sigma_{FLY}^2+ry\sigma_{FL}^2+rl\sigma_{FY}^2+ryl\sigma_{F}^2$
Families × locations	(n-1)(l-1)	$\sigma_{\varepsilon}^2+y\sigma_{\gamma}^2+r\sigma_{FLY}^2+ry\sigma_{FL}^2$
Families × reps/(L)	l(n-1)(r-1)	$\sigma_{\varepsilon}^2+y\sigma_{\gamma}^2$
Families × years	(n-1)(y-1)	$\sigma_{\varepsilon}^2+ r\sigma_{FLY}^2+rl\sigma_{FY}^2$
Families × locations × years	(n-1)(l-1)(y-1)	$\sigma_{\varepsilon}^2+ r\sigma_{FLY}^2$
Families × years × reps(L)	l(n-1)(y-1)(r-1)	σ_{ε}^2

Table 4.5. Means, standard errors and ranges of seed yield and its components in parents and progeny half-sib families.

Variables	Year	Site‡	Parent			Offspring		
			Mean	SE§	Range	Mean	SE	Range
Inflorescence prolificacy rating†	2012	Pks	6.31	0.24	1.00-9.00	5.17	0.14	2.00-8.00
		Stw	6.29	0.21	1.00-9.00	5.40	0.16	3.00-8.00
	2013	Pks	6.24	0.25	1.00-9.00	6.17	0.19	2.00-9.00
		Stw	6.08	0.24	1.00-9.00	5.59	0.18	2.00-8.00
	Combined		6.23	0.12	1.00-9.00	5.59	0.09	2.00-9.00
Seed set (%)	2012	Pks	21.58	1.91	0.73-63.69	37.66	2.23	1.83-86.29
		Stw	27.37	2.34	0.00-85.55	44.28	2.40	4.16-89.54
	2013	Pks	18.06	0.02	0.14-62.78	30.01	2.07	1.38-68.89
		Stw	29.41	2.34	0.20-82.14	24.63	2.00	0.10-73.29
	Combined		24.11	1.07	0.00-85.55	34.15	1.17	0.10-89.54
Seed yield (kg ha^{-1})	2012	Pks	152.23	16.29	1.26-742.44	198.26	9.50	31.75-373.86
		Stw	114.39	11.23	0-419.67	165.75	9.10	4.31-403.18
	2013	Pks	10.54	1.54	0-70.75	23.47	3.65	0-136.30
		Stw	8.05	0.95	0-39.37	7.80	0.84	0.33-33.61
	Combined		71.30	6.16	0-742.44	98.82	5.94	0-403.19

† Rating scale was 1-9, where 1 indicated the least inflorescences while 9 for the greatest inflorescence prolificacy.
‡ Sites are Stw = Stillwater, Pkn = Perkins.
§ Standard error of the group mean.

Table 4.6. Test of fixed effects due to location, and expected mean squares due to random effects of various sources for inflorescences prolificacy, seed set percentage and seed yield among half-sib families combined across locations (Stillwater and Perkins) in 2012.

Sources	Df	Inflorescence prolificacy†	Seed set (%)	Seed yield (kg ha^{-1})
		Test of fixed effects (F values)		
Location (L)	1	1.53	3.73	10.16*
		Expected mean squares		
Family (F)	24	4.16*	0.11*	23960.13*
F × L	24	1.26	0.04*	3902.04
Rep/L	4	1.81	0.04	12709.81*
F × Rep/L	96	1.16*	0.02*	2513.33*

*Significant at the 0.05 probability level.

† Rating scale was 1-9, where 1 indicated the least inflorescences while 9 for the greatest inflorescence prolificacy.

Table 4.7. Test of fixed effects due to location, and expected mean squares due to random effects of various sources for inflorescences prolificacy, seed set percentage and seed yield among half-sib families combined across locations (Stillwater and Perkins) in 2013.

Sources	Df	Inflorescence prolificacy†	Seed set (%)	Seed yield (kg ha^{-1})
		Test of fixed effects (F values)		
Location (L)	1	4.59*	3.36	13.25*
		Expected mean squares		
Family (F)	24	5.67*	0.09*	910.22*
F × L	24	2.81	0.03*	694.42*
Rep/L	4	2.57	0.01	1787.60*
F × Rep/L	96	1.74*	0.02*	336.71*

*Significant at the 0.05 probability level.

† Rating scale was 1-9, where 1 indicated the least inflorescences while 9 for the greatest inflorescence prolificacy.

Table 4.8. Test of fixed effects due to location and year, and expected mean squares due to random effects of various sources for inflorescences prolificacy, seed set percentage and seed yield among half-sib families combined across locations (Stillwater and Perkins) and years (2012 and 2013).

Sources	Df	Inflorescence prolificacy†	Seed set (%)	Seed yield (kg ha^{-1})
		Test of fixed effects (F values)		
Location (L)	1	0.81	0.06	7.99*
Year (Y)	1	9.20*	45.33*	199.86*
L × Y	1	6.45*	9.55*	3.32
		Expected mean squares		
Family (F)	24	6.97*	0.170*	14483.50*
F × L	24	2.15	0.048*	3060.98*
Rep/L	4	2.33	0.007	3986.73*
F × Y	25	3.81*	0.085*	93009.25*
F × L × Y	25	2.34*	0.038*	1686.89
F × Y × Rep/L	196	1.46*	0.020*	1610.44*

*Significant at the 0.05 probability level.

† Rating scale was 1-9, where 1 indicated the least inflorescences while 9 for the greatest inflorescence prolificacy.

Table 4.9. Narrow-sense heritability estimates for seed yield and its components in bermudagrass based on component of genetic variation among half-sib families.

Variables	2012†	2013†	Combined‡
Inflorescence prolificacy	0.70	0.50	0.55
Seed set (%)	0.61	0.63	0.78
Seed yield (kg ha^{-1})	0.84	0.23	0.18

†Estimates were based on the single year data across locations.
‡Estimates were based on the two-year data across locations.

Table 4.10. Narrow-sense heritability estimates and their standard errors (in parentheses) for inflorescences prolificacy, seed set percentage and seed yield in 2012 and 2013 based on parent-offspring regression.

Traits	Stillwater†	Perkins‡	Stillwater†	Perkins‡
	2012	2012	2013	2013
Inflorescence prolificacy	0.30*(0.15)	0.53*(0.14)	0.21(0.16)	0.33(0.18)
Seed set (%)	0.76*(0.28)	0.33(0.21)	0.66*(0.27)	0.41*(0.20)
Seed yield (kg ha^{-1})	0.26*(0.13)	0.68*(0.18)	-0.10(0.13)	1.38(0.88)

†Data for half-sib families were at Stillwater site, and for parents at Perkins site.
‡Data for half-sib families were at Perkins site, and for parents at Stillwater site.
*Significant at the 0.05 probability level.

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Figure 4.1. Design for planting the progeny plants of one half-sib family within each plot. Each plant was planted in the center of one square feet. '×' locates one grown progeny plant representing the half-sib family.

Figure 4.2. Design for planting the maternal plants within each subplot. '×' locates one grown maternal clone. Four plants were planted in one plot, with each one in the center of 4 feet $\times$2.5 feet area.

Figure 4.3. Average temperatures (line) and total rainfall (filled line) at Perkins and Stillwater experimental sites during 2012 and 2013.

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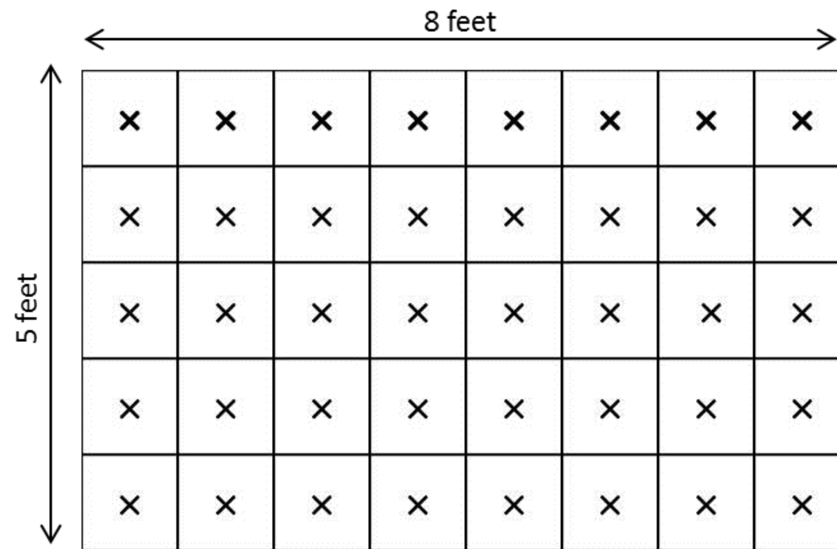

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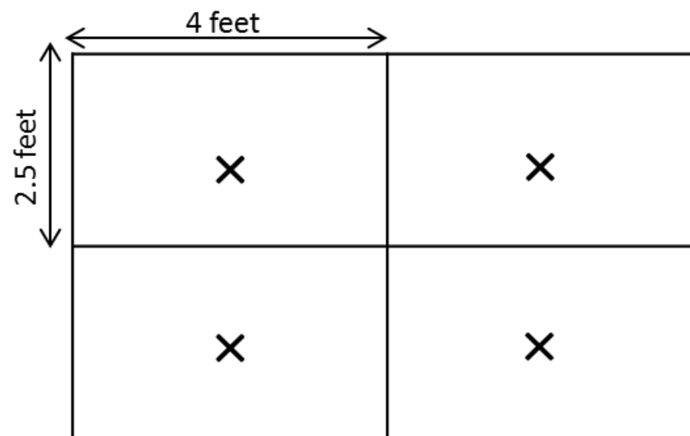

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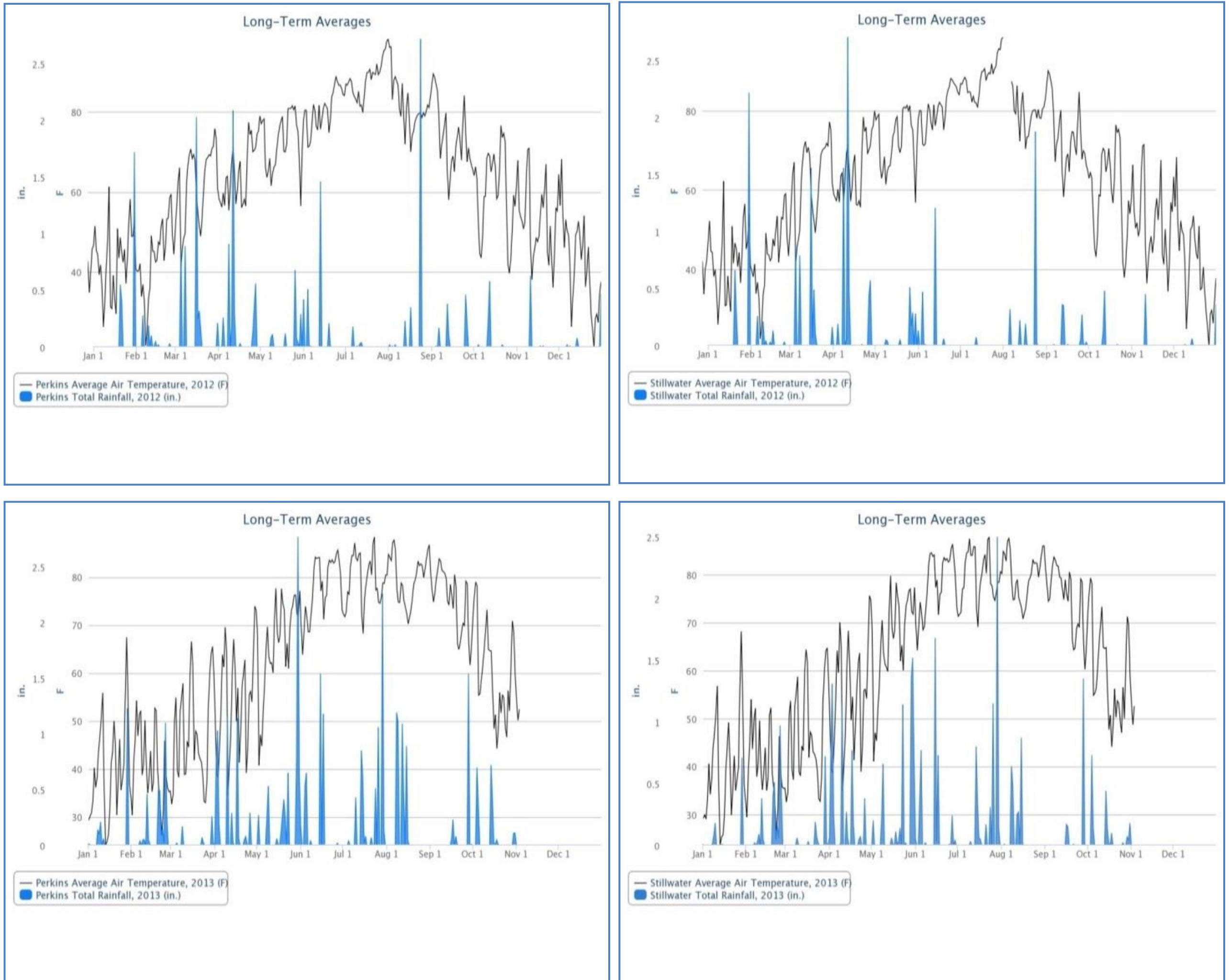

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